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The importance of arginase for vascular dysfunction in patients with glucose and lipid abnormalities

by

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To my family

"Do not go where the path may
lead, go instead where there is
no path and leave a trail."

Ralph Waldo Emerton

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Karolinska University Hospital, Stockholm, Sweden

The importance of arginase for vascular dysfunction in patients with glucose and lipid abnormalities

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Abstract

Introduction
Endothelial dysfunction is one important mediator behind atherosclerosis. Maintained bioavailability of nitric oxide (NO) is critical to keep the fragile balance of endothelial function. Reduced NO arise from reduced production or increased elimination of NO. Arginase is an enzyme which metabolizes the substrate, L-arginine, which is used to produce NO. By competitive inhibition arginase may result in reduced NO-bioavailability. Several risk factors for atherosclerosis such as diabetes mellitus and hypercholesterolemia are known to upregulate arginase expression and activity. Furthermore, experimental research has demonstrated beneficial effects of arginase inhibition. However, the functional significance of arginase in regulation of endothelial dysfunction in patients with cardiovascular disease is unknown.

Aim
To evaluate the significance of arginase inhibition on endothelial function in patients with glucose and lipid abnormalities.

Study I
Forearm blood flow was determined during the administration of serotonin and sodium-nitroprusside to evaluate endothelium-dependent (EDV) and endothelium-independent (EIDV) vasodilatation, respectively, before and after 120 minutes intra-arterial (i.a.) administration of the arginase inhibitor N^{ω}-hydroxy-L-arginine (nor-NOHA) in patients with coronary artery disease (CAD) with and without type 2 diabetes mellitus and in control subjects. Administration of nor-NOHA increased EDV in patients with CAD and diabetes and in patients with CAD alone via a NOS-dependent mechanism. Nor-NOHA did not affect EDV in control subjects. EIDV increased slightly in the CAD and diabetes group following nor-NOHA.

Study II
Microvascular endothelial function was evaluated using laser Doppler flowmetry before and after 120 minutes i.a administration of nor-NOHA. EDV was reduced in subjects with type 2 diabetes mellitus and microvascular dysfunction compared to healthy subjects. Administration of nor-NOHA reversed the impairment of microvascular endothelial function in the diabetes group, but not in the control group. The levels of amino acids reflecting arginase relative activity compared to NO synthase were significantly higher in subjects with diabetes mellitus, suggesting a higher arginase activity in this group.

Study III
Flow-mediated vasodilatation (FMD) of the brachial artery was evaluated in patients with CAD without and with type 2 diabetes mellitus before and after 20 minutes of forearm ischemia and 20 minutes of reperfusion in a cross-over protocol randomized to either nor-NOHA or NaCl. FMD was reduced in CAD patients following ischemia-reperfusion (IR) and administration of NaCl. Administration of nor-NOHA prevented the decrease in FMD after IR. In the group with CAD and diabetes, FMD following IR was significantly greater during administration of nor-NOHA than during administration of NaCl.

Study IV
EDV and EIDV was determined in patients with familial hypercholesterolemia during relative low and high cholesterol levels compared to healthy control subjects before and after arginase inhibition. Baseline EDV did not differ between the groups. All groups increased their EDV in response to nor-NOHA. The improvement in EDV was greater among patients with familial hypercholesterolemia subjects regardless of their cholesterol level or statins use. EIDV was not affected by nor-NOHA.

Conclusions
Collectively, these results demonstrate the importance of arginase for the regulation of endothelial function in patients with CAD, type 2 diabetes mellitus, and hypercholesterolemia. Arginase is a promising therapeutic target in the future treatment of endothelial dysfunction in patients with CAD, type 2 diabetes mellitus, or hypercholesterolemia.

SAMMANFATTNING

Bakgrund
Åderförkalkning är idag den vanligaste orsaken till död i världen. Endoteldysfunktion är ett förstadium till åderförkalkning och börjar tidigt i sjukdomsutvecklingen. Kväveoxid (NO) är en viktig molekyl som produceras av endotelet och påverkar graden av inflammation, proppbildning och kärltonus. Många riskfaktorer för åderförkalkning har visat sig minska biotillgängligheten av NO. Arginas är ett enzym i endotelcellerna som metaboliserar arginin, dvs samma substrat som används för att bilda NO. Många experimentella studier har visat att ökad arginasaktivitet kan bidra till endoteldysfunktion vid ateroskleros, diabetes och hyperlipidemi.

Målsättning
Att undersöka betydelsen av arginas vid endoteldysfunktion hos patienter med kranskärlssjukdom samt glukos- och lipidrubbningar.

Studie I
Endotelfunktion i underarmen bestämdes före och efter 120 min i.a. administration av arginashämmaren N^{ω}-hydroxy-L-arginin (nor-NOHA) hos patienter med CAD med och utan typ 2 diabetes samt friska kontroller. Administrering av nor-NOHA förbättrade endotelfunktionen hos patienter med CAD både med och utan diabetes men inte hos friska kontroller. Patienterna med diabetes hade signifikant bättre svar efter nor-NOHA jämfört med CAD patienter utan diabetes. Förbättringen i endotelfunktionen var beroende av det NO-producerande enzymet NO-syntas.

Studie II
Mikrovaskulär endotelfunktion undersöktes hos patienter med typ 2 diabetes och mikrovaskulär dysfunktion före och efter 120 min i.a. administrering av nor-NOHA. Diabetesgruppen hade en sänkt endotelfunktion jämfört med friska kontroller. Efter administration av nor-NOHA förbättrades endotelfunktionen i diabetesgruppen till samma nivå som hos friska kontroller. Endotelfunktionen hos friska kontroller påverkades inte av interventionen. Aminosyror som speglar aktiviteten av arginas i jämförelse med NO-syntas var förhöjda i diabetesgruppen.

Studie III
Flödesmedierad vasodilatation (FMD) av radialisartären bestämdes före och ischemi-reperfusion (IR) i underarmen hos patienter med kranskärlssjukdom med och utan typ 2 diabetes i ett cross-over protokoll där patienterna randomiserades i.a. infusion av till nor-NOHA eller NaCl. FMD minskade efter IR hos patienter med kranskärlssjukdom utan diabetesvid administrering av NaCl. Denna försämring motverkades av administrering av nor-NOHA. Även hos patienter med diabetes var FMD förbättrad vid administrering av nor-NOHA jämfört med NaCl efter IR.

Studie IV
Endotelfunktion bestämdes hos patienter med familjär hyperkolesterolemi vid höga och låga kolesterolnivåer samt hos friska kontroller före och efter arginasblockad. Basal endotelfunktion skilde sig inte åt mellan grupperna. Alla grupperna förbättrades i endotelfunktion efter administration av nor-NOHA. Förbättringen var signifikant större hos patienterna med familjär hyperkolesterolemi oavsett om dom hade lipidsänkande behandling eller ej. Endoteloberoende vasodilatation påverkades ej av arginasblockad.

Konklusion
Vi har visat att hämning av arginas spelar en betydelsefull roll för reglering av endotelfunktionen hos patienter med kranskärlssjukdom, diabetes och FH. Resultaten i denna avhandling talar för att hämning av arginas är en lovande terapi mot endoteldysfunktion hos patienter med utvecklad kranskärlssjukdom samt vid glukos- och lipidrubbningar.

List of Abbreviations

BH_4	Tetrahydrobiopterin
CAD	Coronary artery disease
EDV	Endothelium-dependent vasodilatation
EIDV	Endothelium-independent vasodilatation
FBF	Forearm blood flow
FH	Familial hypercholesterolemia
FMD	Flow-mediated vasodilatation
HDL	High-density lipoprotein
i.a.	Intra-arterial
IR	Ischemia-reperfusion
LDF	Laser Doppler flowmetry
LDL	Low-density lipoprotein
L-NMMA	L-N^G-monomethyl arginine
LOX-1	Lectin-like oxidized low-density lipoprotein receptor 1
NO	Nitric oxide
Nor-NOHA	N^{ω}-hydroxy-nor-L-arginine
NOS	Nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
O_2^-	Superoxide
$ONOO^-$	Peroxynitrite
OxLDL	Oxidized low-density lipoprotein
p38MAPK	p38 mitogen-activated protein kinase
PGI_2	Prostacyclin
RBC	Red blood cell
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
SNP	Sodium nitroprusside
UKPDS	UK Prospective Diabetes Study

List of Publications

This thesis is based on the following published articles:

I.

Alexey Shemyakin, **Oskar Kövamees**, Arnar Rafnsson, Felix Böhm, Peter Svenarud, Magnus Settergren, Christian Jung, John Pernow.

Arginase inhibition improves endothelial function in patients with coronary artery disease and type 2 diabetes

Circulation. 2012;126(25):2943-50.

II.

Oskar Kövamees, Alexey Shemyakin, Antonio Checa, Craig E Wheelock, Jon O Lundberg, Claes-Göran Östenson, John Pernow.

Arginase inhibition improves microvascular endothelial function in patients with type 2 diabetes mellitus.

J Clin Endocrinol Metab. 2016:101(11):3952-3958

III.

Oskar Kövamees, Alexey Shemyakin, John Pernow.

Effect of arginase inhibition on ischemia-reperfusion injury in patients with coronary artery disease with and without diabetes mellitus.

PLoS One. 2014;9(7):e103260.

IV.

Oskar Kövamees, Alexey Shemyakin, Mats Eriksson, Bo Angelin, John Pernow.

Arginase inhibition improves endothelial function in patients with familial hypercholesterolaemia irrespective of their cholesterol levels.

J Intern Med. 2016;279(5):477-84.

INTRODUCTION AND BACKGROUND

Cardiovascular disease and atherosclerosis

Cardiovascular disease is the most common cause of death in the world today. Approximately 80% of deaths due to cardiovascular causes are due to diseases related to atherosclerosis such as myocardial infarction or stroke (1). Atherosclerosis is a chronic and systemic condition of inflammation and lipid accumulation in the arterial wall (2). The disease is slowly progressive and is built up over decades as illustrated in Figure 1. Eventually, atherosclerosis results in formation of a plaque which may obstruct the blood flow and thereby cause ischemia to its distal tissue. The plaque may become unstable over time and rupture which triggers formation of a thrombus that may cause total occlusion of blood flow resulting in ischemia and cell death to either the heart (myocardial infarction) or brain (stroke) depending on the vascular territory of the plaque (3).

The incidence of cardiovascular disease is increasing worldwide especially in low-and middle-income countries. The WHO estimates that 75% of cardiovascular deaths occur in these countries (1). Worldwide approximately 14 million deaths are due to the complications of atherosclerosis such as coronary artery disease (CAD) and stroke (1). It is important to note that 46% of people dying from cardiovascular disease are below 70 years of age, which is a highly productive time in people's lives (4).

INTERHEART, a case-control study which compared patients with acute myocardial infarction with control subjects, found that nine risk factors are responsible for 90% of the events (5). These include smoking, elevated blood lipids (hyperlipidemia), systemic hypertension (hypertension), diabetes mellitus, lack of regular physical activity, adverse psychosocial conditions, and abdominal obesity. Analyses of changes in risk factors and medical interventions in populations over time have generated interesting data. The increased mortality from CAD in the population of Beijing between 1984 and 1999 was explained mainly by an increase in mean cholesterol and an increased prevalence of diabetes mellitus (6). During the same time period, the age-adjusted mortality from CAD decreased in the US, a change explained partly by a reduction of risk factors, i.e reductions in cholesterol, blood pressure, smoking prevalence, and physical inactivity. In contrast, the risk factors obesity and diabetes mellitus increased in prevalence (7).

Diabetes mellitus and hyperlipidemia are considered to be two very important risk factors for the development of cardiovascular disease (8). The exact mechanisms involved in the pathogenesis of atherosclerosis and the development of CAD are still under investigation. Evidence suggests that endothelial dysfunction is closely linked to these risk factors, occurs early in the process of atherosclerosis and may amplify the progression and prognosis of the disease (Figure 1) (9, 10).

Diabetes mellitus

Diabetes mellitus is a chronic condition characterized by an elevated glucose concentration in the blood. The WHO has defined diabetes mellitus as a fasting plasma glucose $\geq$ 7.0 mmol/l or plasma glucose $\geq$ 11.1 mmol/l two hours after an oral glucose load (75g). There are different

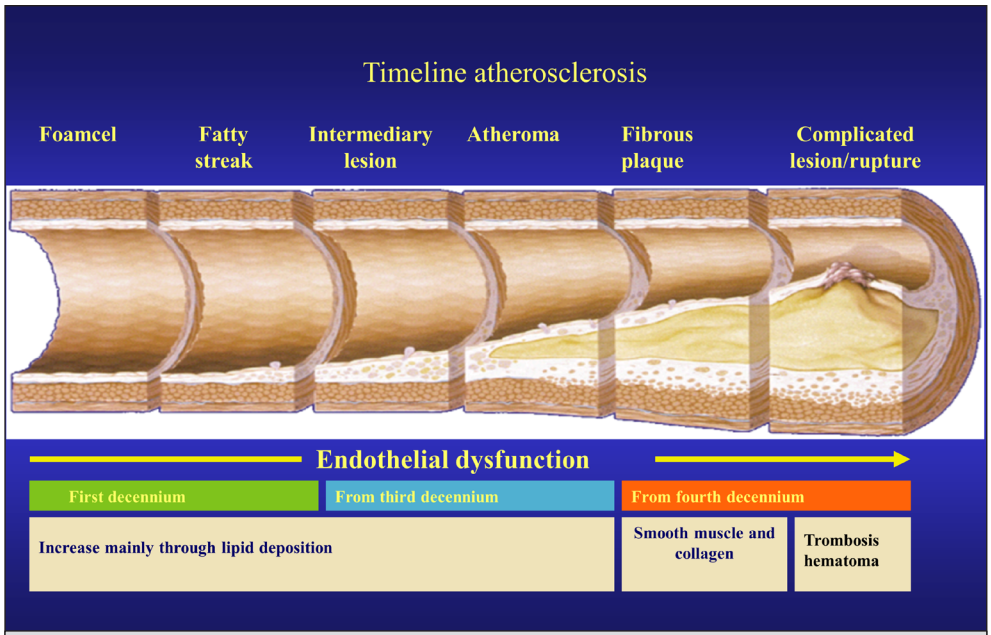

Figure 1. The atherosclerosis process. Timeline over the progression of atherosclerosis. Endothelial dysfunction is an early mediator of atherosclerosis and is maintained throughout the disease. With permission from Professor Joep Perk who adapted it from Stary HC et al. Circulation. 1995;92:1355-1374 (3).

subgroups of diabetes mellitus, mainly type 1, type 2, gestational, and other specific types. Prediabetes is considered a condition of elevated plasma glucose, although not high enough to be classified as type 2 diabetes mellitus. Prediabetes individuals have an increased risk of developing type 2 diabetes and cardiovascular disease (11). According to the WHO definitions, impaired fasting glucose is the occurrence of plasma glucose between 6.1-6.9 mmol/l under fasting conditions while the individual still retains a normal response to oral glucose tolerance test (<7.8 mmol/l). Impaired glucose tolerance is defined as fasting glucose value <7.0 mmol/l in addition to plasma glucose value between 7.8-11.0 mmol/l after oral glucose tolerance test. The progression from impaired glucose tolerance to type 2 diabetes mellitus has been shown to be 90% over 20 years in the absence of therapeutic intervention (12).

Today there is an epidemic of diabetes. The International Diabetes Federation estimates that there were approximately 415 million patients suffering from diabetes in 2015 (8.8% of the world population). Of those, approximately 90% consist of type 2 diabetes. The estimated cost is about 673 billon US dollars per year (12% of global health expenditure) (11). In addition, the prevalence of diabetes mellitus is increasing. Estimations project that by 2040 the world-wide prevalence will be 642 million people (10.4% of the world population) and will account for a major increase in the disease burden for health care systems and the economic cost to society. The epidemiology of the condition is also changing. Whereas the highest incidence and prevalence used to be seen in high-income countries, now about 75% of the patients exist in low- and middle-income countries (11). In Sweden (a high-income country), the prevalence of diabetes mellitus has increased by 61%, to approximately 352 400 patients (4.4% of the national population), between the years 2006-2013, which is probably because of longer average life expectancy since the incidence has been stable (13).

The pathological process of type 2 diabetes mellitus often starts with decreased insulin sensitivity which in combination with inability of the pancreas to produce enough insulin yields a reduced glucose uptake in skeletal muscle and other tissues. This in turn results in increased levels of glucose in the blood. The onset of type 2 diabetes mellitus occurs commonly in adulthood and is silent and gradual, meaning it is common for the disease to remain undiagnosed for many years. Genetic, environmental, and lifestyle factors have been described which contribute to the development of type 2 diabetes mellitus (11, 14). Most genes associated to type 2 diabetes mellitus are linked to insulin production or secretion, or have so far an unclear function. Among the life style risk factors, overweight and/or low levels of physical activity are strongly associated with the development of type 2 diabetes mellitus (11, 15). Moderate or high levels of physical activity, weight reduction and coffee intake have been shown to mediate protective effects (16-18).

The treatment of type 2 diabetes mellitus is focused on lifestyle changes and medication, with the drug of choice being metformin (19). Recently, there has been a debate whether intensive treatment with classic glucose lowering drugs improves cardiovascular outcomes in patients with diabetes. Recently developed pharmacological agents like glucagon-like peptide 1 agonists and sodium-glucose co-transporter-2 inhibitors have been shown to be associated with a reduction of a composite outcome including death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke (20, 21). However, evidence suggests that the classical paradigm of a glucose lowering regime is not sufficient to reduce cardiovascular mortality. The UK Prospective Diabetes Study (UKPDS 33) showed a reduced relative risk of developing microvascular complications in the intensively treated group compared to standard treatment (22). By contrast, there were no change in mortality and a non-significant reduction in myocardial infarctions ($p=0.052$). A follow-up study, UKPDS 34, showed a reduced frequency of cardiovascular events (23). Hence, newly diagnosed diabetes seems to gain from intensive treatment. However, for patients that have had diabetes for a longer time the evidence for beneficial effect on cardiovascular outcome with intensive glucose lowering treatment are less clear (24). The limited effect of glucose lowering therapy alone on cardiovascular complications suggests that new mechanisms behind vascular complications in patients with type 2 diabetes mellitus need to be identified and explored. In the following pages this thesis will focus on type 2 diabetes mellitus which henceforth will be referred to as diabetes.

The link between diabetes and cardiovascular disease

Diabetes is associated with microvascular and macrovascular complications. Microvascular complications include nephropathy (vascular damage in the kidneys), retinopathy (vascular damage in the eyes), and neuropathy (mainly in peripheral nerves, causing sensations of numbness, pain, or buzzing in extremities). These complications may eventually progress to blindness, renal failure, and amputation of extremities. Macrovascular complications refer to myocardial infarction, stroke, or intermittent claudication. Cardiovascular disease is the main cause of death in patients with diabetes, with 50% estimated to die from macrovascular complications (25). Among patients with CAD, 30% have been shown to have diabetes and an additional 35% have impaired glucose intolerance (26). Patients with diabetes had three times higher risk for cardiovascular mortality than patients with without diabetes (27, 28). Furthermore, hypertension and hyperlipidemia commonly co-exists with diabetes (29). These findings constitute a clear link between diabetes and cardiovascular disease.

The vasculature

There are three different blood vessels in the human body: arteries transporting blood away from the heart towards the body's organs, veins transporting blood back to the heart, and capillaries in which the exchange of oxygen and nutrients take place at the organ tissue level. The main objective of the vasculature is to transport oxygenated blood, carried by red blood cells, to distal tissues where oxygen and waste products from cell metabolism are exchanged. These red blood cells are then transported back to the lungs to be re-oxygenated. If the red blood cells are the cargo trucks of the body the vasculature can be considered the highway. A blood vessel can be divided anatomically into three parts, the adventitia, the media and the endothelium. The external layer, the adventitia, is mostly constructed from connective tissue whereas the media consists mainly from smooth muscle cells. The vascular endothelium is a monolayer of cells, separating the circulating blood from the vessel wall. The endothelial layer is estimated to contain 10^{13} cells and, in the human, if spread out flat hypothetically would cover 1000 m^2 (30). For many years the vascular endothelium was only considered to be a semi-permeable barrier with the sole purpose of keeping the blood circulating without unnecessary leakage. This simple concept of endothelial function has changed dramatically over the last 30 years and is the focus of this thesis.

The endothelium and endothelial function

A new area of research was introduced with the discovery that vasodilatation could be elicited by an endothelium-derived factor (31). With time, many other critical capabilities were discovered and the endothelium gained acceptance as a key regulator of vascular homeostasis. The endothelium produces substances affecting inflammation, thrombosis, and vascular tone. It is crucial that the endothelium maintains a proper balance between substances affecting these properties. Endothelial dysfunction is an imbalance of substances produced by the endothelium leading to disturbed homeostasis and eventually atherosclerosis. Nitric oxide (NO) is a molecule, produced by the endothelium, which mediates vasodilation, and has both anti-inflammatory, and anti-coagulant properties. However, during conditions of reduced bioavailability of NO, the positive actions mediated by this reactive molecule are diminished (32), initiating endothelial dysfunction (Figure 2). Taken together, the endothelium plays an important part in maintaining homeostasis by balancing vascular tone, inflammation, and coagulation.

Endothelial dysfunction is central for the pathogenesis of atherosclerosis (33) and its risk factors diabetes (34) and hyperlipidemia (35). Endothelial dysfunction is associated with clinical events in patients with atherosclerosis and diabetes (9, 36). As illustrated in Figure 1, evidence suggests that endothelial dysfunction occurs early in the process of atherosclerosis and may amplify the progression and the poor prognosis of the disease (9, 10).

As mentioned above, the pathology behind endothelial dysfunction is a reduction in the bioavailability of NO. In the endothelium, NO is produced together with citrulline from L-arginine by the enzyme endothelial NO synthase (eNOS) (37). Initially, NOS hydrolyses L-arginine to N-hydroxy-L-arginine, which is oxidized to citrulline and NO in a second step (38). One important mechanism of endothelial dysfunction is thus the lack of substrate for eNOS, L-arginine. There are three main forms of NOS enzymes. These are eNOS, neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS and nNOS are constitutively expressed by a

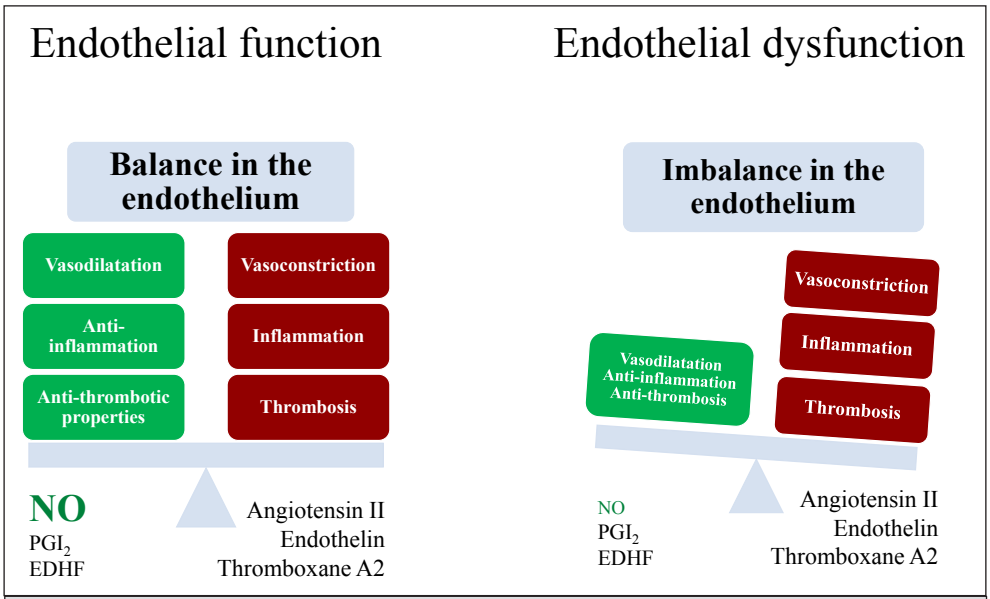

Figure 2. A schematic figure describing in general the delicate balance between endothelial factors regulating endothelial function. Nitric oxide (NO) is responsible for multiple positive actions in the endothelium. If NO bioavailability decreases, there is increased activation of vasoconstriction, inflammation and thrombosis. EDHF, endothelium-derived hyperpolarizing factor; NO, nitric oxide; PGI2; prostacyclin.

wide range of cells. eNOS is mainly expressed in endothelial cells whereas nNOS mainly is expressed by neurons. The activity of the constitutive isoform is regulated by the intracellular concentration of Ca^{2+}. During conditions of increased Ca^{2+} the co-factor calmodulin increases its affinity for eNOS/nNOS, hence facilitating the electron transport which is crucial for the function of the enzyme (39). Furthermore, the activity of eNOS is regulated by phosphorylation, targeting mainly the sites Ser^{1177} and Thr^{495}, yielding additional and reduced activity, respectively. In the resting state, Ser^{1177} is usually not phosphorylated, however, in response to insulin, estrogen, sheer stress, or bradykinin it becomes phosphorylated by the activation of different kinases. Phosphorylated Ser^{1177} mediates its response by increasing the electron transport and increasing Ca^{2+}-sensitivity (40, 41). On the other hand, phosphorylation of Thr^{495} inhibits the function of eNOS (39). iNOS is expressed by inflammatory cells and is regulated at the transcriptional level (42) and is Ca^{2+}-independent (43).

Decreased bioavailability of NO, defined as reduced concentration of NO available for mediating a biological response, is caused either by reduced production or increased inactivation of NO. The reasons behind inactivation and reduced production are multifactorial (Figure 3). Examples of molecular mechanisms are decreased co-factors, phosphorylation, production of ROS, and decreased substrate (44). Furthermore, one or several of these mechanisms may lead to the uncoupling of eNOS. Under conditions of oxidative stress and reduced levels of the important substrate L-arginine or the co-factor tetrahydrobiopterin (BH_4), eNOS may produce the ROS superoxide (O_2^-) from O_2 instead of NO from L-arginine (44). ROS might also be produced from other sources such as xanthine oxidase, NADPH

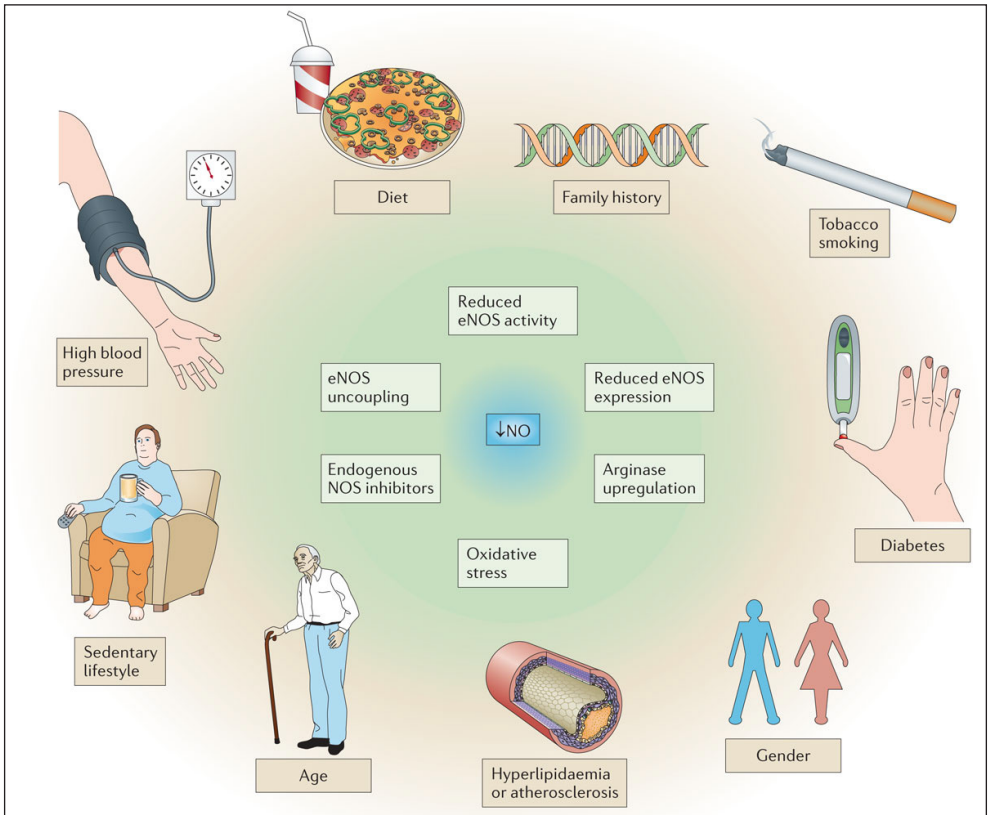

Figure 3. Intact nitric oxide (NO) signalling is vital for homeostasis in the cardiovascular system through the regulation of vascular tone, platelet aggregation and cardiac function. Several risk factors (brown boxes) for cardiovascular disease promote processes (green boxes) that ultimately lead to a decrease in NO bioavailability. This occurs through enhanced NO degradation, attenuation of NO synthesis or desensitization of downstream NO signalling. eNOS, endothelial NOS; NOS, nitric oxide synthase. Figure and legend reproduced from (159). Permission obtained from Nature Publishing Group.

oxidases, and the mitochondrial respiratory chain (45). NO and O_2^- may react and form peroxynitrite ($ONOO^-$), an extremely reactive and cytotoxic compound (44). It is rapidly protonated and forms peroxynitrous acid. On exiting the cell, due to the concentration gradient, peroxynitrous acid undergoes a cleavage and forms hydroxyl and nitric dioxide two highly reactive oxygen species. Both of these compounds play a central role in oxidative stress leading to reduction of BH_4, further uncoupling eNOS producing more ROS (44). Oxidative stress therefore reduces active NO by: 1) superoxide reacting with NO itself, decreasing the free concentration of NO and 2) decreasing the cofactors, thereby mediating eNOS uncoupling and more ROS production.

Diabetes, hypercholesterolemia, and endothelial dysfunction

As mentioned above diabetes is associated with a substantial risk for cardiovascular disease (8). Existing data indicate that endothelial dysfunction is an important mediator in the

development of cardiovascular complications in diabetes (36) and that these complications are a major cause of mortality among patients with diabetes (46). Suggested mechanisms behind the development of cardiovascular disease in patients with diabetes include pro-inflammatory activation, reduced fibrinolytic capacity, and reduced platelet function (47, 48). Important triggers of these changes are hyperglycemia, hyperlipidemia, and oxidative stress leading to endothelial dysfunction (34). Hyperglycemia, insulin-resistance and elevated free fatty acids have been show to inhibit NO formation (34) and increase ROS formation (49). The vasoconstrictor and pro-inflammatory peptide endothelin-1 is produced in hyperinsulinemia (50) and hyperglycemia (51). Taken together, impaired NO-mediated vasodilatation and increased endothelin-1 has been observed in patient with diabetes (52). These processes influence the balance and function of the endothelium to promote vasoconstriction and inflammation.

Hyperlipidemia in the form of hypercholesterolemia is an independent risk factor for cardiovascular disease and is also common in patients with diabetes (53). It is described that patients with hypercholesterolemia have impaired endothelial function both in the absence and presence of developed CAD (35, 54, 55). Accordingly, endothelial dysfunction is an early event observed in patients with familial hypercholesterolemia (FH) (56-58). Elevated low-density lipoprotein (LDL) cholesterol is an important risk factor for the development of atherosclerosis and CAD (53). FH is a genetic disorder characterized by high levels of total and particularly LDL cholesterol resulting in the premature development of atherosclerosis and its vascular complications including CAD (59). There is no elevation in the absolute number of LDL particles in patients with diabetes compared to patients without diabetes (60). However, the LDL particle in patients with diabetes is more dense (61), is more likely to undergo oxidative alterations (60) and is more easily taken up by smooth muscle cells and monocytes (62). Further, LDL particles in patients with type 2 diabetes are glycated (63). These qualitative modifications of LDL amplify the atherosclerotic process in diabetes (64).

Reperfusion injury

A clinical complication of coronary artery atherosclerosis is plaque rupture, coronary artery occlusion, and development of acute myocardial infarction. Standard treatment of ST-elevation myocardial infarction (a complete occlusion of blood flow in a coronary artery supplying the myocardium) is based on rapid reperfusion of the occluded coronary artery using either percutaneous coronary intervention or thrombolysis. Such treatments have led to limitation of myocardial injury and improved survival. However, reperfusion *per se* is associated with cellular injury contributing to the final vascular and myocardial injury (65). Ischemia-reperfusion (IR) injury has gained much interest as a main element of the total damage resulting from an acute myocardial infarction. Experimental studies have demonstrated that it is possible to preserve up to 50% of the area at risk in the myocardium by reducing IR injury (66). The mechanisms behind the IR injury involve several factors such as endothelial dysfunction, increased pro-inflammatory activity, increased formation of ROS, elevation of intracellular Ca^{2+}, and opening of mitochondrial permeability transition pores (65), all of which contribute to the total myocardial injury. Endothelial dysfunction is suggested to be one key event in the development of IR (67) as the bioavailability NO is of importance for the protection against IR injury (32). Administration of NO-donor increased the bioavailability of NO and decreased infarct size in mouse hearts. The reduction in infarct size was abolished by co-administration of an NO-scavenger (68), emphasizing the

importance of NO for cardioprotection. As arginase regulates the bioavailability of NO and arginase inhibition has been shown to decrease infarct size in the experimental setting (66, 69), inhibition of arginase might be of importance for cardioprotection in IR injury. However, no studies have evaluated arginase inhibition in the clinical setting. Many interventions have been suggested to reduce IR injury, and experimental studies have been promising. However, the translation into the clinic has been, in general, disappointing and many clinical studies have failed to demonstrate a reduction in infarct size in patients with ST-elevation myocardial infarction. Therefore, there is currently no intervention to reduce IR injury in clinical use.

Arginase and cardiovascular disease

As described above, the mechanisms behind the reduction of the bioavailability of NO are complex, including both decreased production and increased elimination of NO. One mechanism that has generated much interest is increased activity of the enzyme arginase, which metabolizes the NO substrate L-arginine to ornithine and urea (Figure 4). Arginase

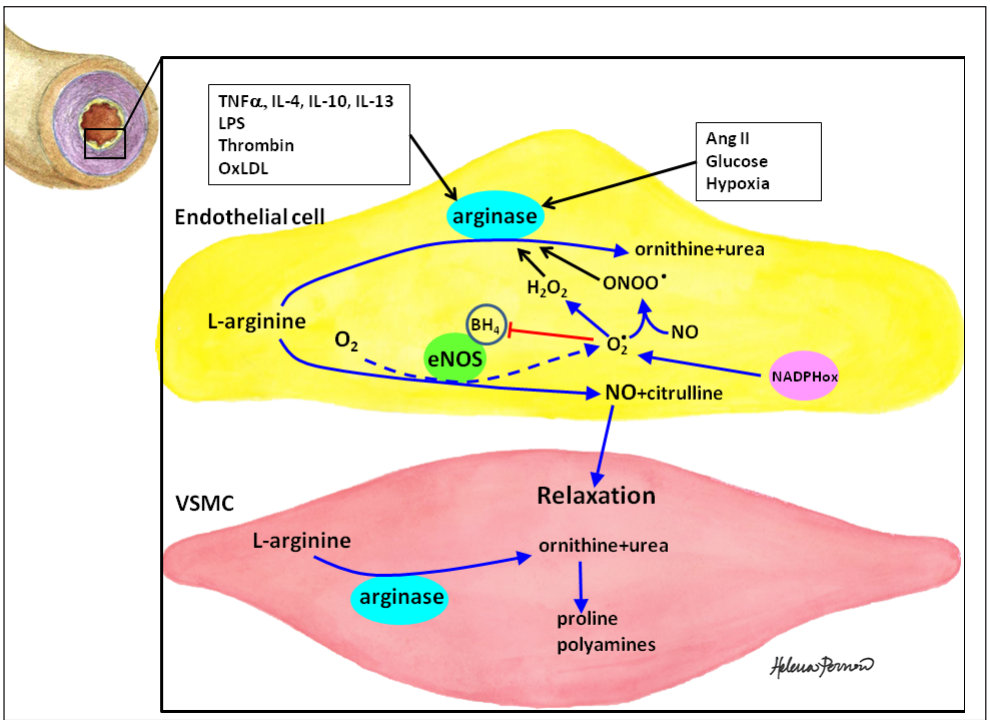

Figure 4. A schematic illustration of the interaction between eNOS and arginase. Arginase metabolizes the same substrate as eNOS, L-arginine, and is up-regulated in response to different stimuli (white boxes). During conditions of reduced availability co-factors or substrate eNOS produce ROS which further could up-regulate arginase and decrease the ability for eNOS to produce NO. Ang II; angiotensin II, eNOS; endothelial nitric oxide synthase; H2O2, hydrogen peroxide; IL, interleukin; LPS, lipopolysaccharide; oxLDL, oxidized low-density lipoprotein; NO, nitric oxide; O2; superoxide, NADPHox, nicotinamide adenine dinucleotide phosphate-oxidase; ONOO, peroxynitrite; TNF-α, tumor necrosis factor- α; VSMC, vascular smooth muscle cells. Reproduced from (73). Permission obtained from Oxford University Press.

is abundant in the liver where it plays a key role in the urea cycle, eliminating ammonia from the amino acid metabolism, but it is also expressed throughout the cardiovascular system (70). Two different isoforms of arginase have been identified. Arginase I is mainly expressed in the liver where it hydrolyses L-arginine as a part of the urea cycle. The other isoform, arginase II, is a mitochondrial protein expressed mostly in extra-hepatic tissues (70). The arginase enzymes share 50% of their amino acids, and their amino acid sequence is the same in areas vital for their enzymatic function (71) although they are coded from two different genes. Recent research has established that both arginase I and II are expressed in the cardiovascular system such as endothelial and vascular smooth muscle cells of blood vessels, and in cardiomyocytes (70, 72). However, the role of arginase in the cardiovascular system is incompletely understood and the expression of arginase differs between species and between different vascular beds (73). Arginase reciprocally regulates NO-production by using the same substrate and thereby reduces the availability of L-arginine for eNOS, reducing NO production and mediates endothelial dysfunction. Furthermore, arginase represses the translation and stability of iNOS (74, 75). Depletion of L-arginine also leads to the uncoupling of eNOS which results in ROS production (76), further enforcing this vicious cycle of ROS and reduced NO. Thus, arginase may contribute to impaired bioavailability of NO both by reducing the production of NO from L-arginine and by facilitating the inactivation of NO via increased formation of ROS. Vascular arginase has been described as being up-regulated by cardiovascular risk factors such as hyperglycemia and LDL cholesterol resulting in increased arginase activity in atherosclerosis, myocardial ischemia and reperfusion and diabetes (66, 77-81).

Expression and activity of arginase

Arginase has gained much interest in the cardiovascular field as a regulator of endothelial function. Multiple factors involved in cardiovascular disease are known to modulate the activity or expression of arginase. Inflammation is of key importance in both atherosclerosis and diabetes. Tumor necrosis factor, interleukin-4, and interleukin-13 have been show to increase arginase activity (82, 83). This is further supported by the fact that arginase activity and expression of arginase I were up-regulated in a mouse model of endotoxin-induced uveitis (84). In humans, patients with inflammatory bowel disease have increased levels of arginase (85). ROS and peroxynitrite have been shown to increase arginase (86-88), suggesting an important regulatory action of oxidative stress. This is of interest considering that uncoupled eNOS produces ROS, which easily reacts with NO and forms peroxynitrite.

Accumulating evidence suggest important links between LDL and arginase. In cell and rat experiments, oxidized LDL (oxLDL) particles have the potential of increasing arginase through activation of the RhoA/ROCK-pathway by upstream activation of lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) (89). The same study observed that inhibition of ROCK reduced the increase in arginase after oxLDL stimulation. Furthermore, oxLDL induced Rho translocation and activation, which could be prevented with a LOX-1 antibody or statins. Rho silencing or Rho-kinase inhibition reduced oxLDL-stimulated arginase II activation (89). Additional studies have demonstrated reduced arginase expression after inhibition of RhoA and ROCK, suggesting that this pathway could be importance in the regulation of arginase (85). In an experimental models of atherosclerosis, e.g. apolipoprotein

E knockout mice, arginase II was increased compared to control animals (90). This is in line with the results of similar work of the same animal model (91, 92). Rabbits fed cholesterol rich diet have increased expression of both isoforms of arginase (93). In humans, it has been demonstrated that maternal hypercholesterolemia in pregnancy is associated with increased arginase expression and activity in the umbilical vein accompanied with reduced eNOS activity (94). Taken together, up-regulation of arginase is present during LDL exposure and one pathway could be activation of LOX-1 and RhoA/ROCK.

Glucose has been shown to increase arginase (95), which is of interest considering the important role of endothelial dysfunction in diabetes. In experimental models of diabetes arginase activity and arginase I expression were increased in aorta (78). Furthermore, the impairment in endothelial function correlated with arginase activity and production of ROS (78). Expression of arginase is increased in aorta and myocardium of rats with diabetes (77). Arginase II is involved in the reduced relaxation of corpus cavernosum in diabetic animals (96) and arginase expression was increased in corpus cavernosum in patients with diabetic erectile dysfunction (97). Reports from one clinical study show an up-regulation in plasma arginase in addition to a reduced NOS activity in patients with diabetes (81). Another recent study observed up-regulation of arginase I in coronary arterioles from patients with diabetes, and that arginase contributes to the reduced NO-production *in vitro* (98). Hence, arginase seems to be of importance in the experimental and possibly in the clinical setting of diabetes. In an experimental model of IR injury, arginase expression was increase in coronary endothelial cell and smooth muscle cells (99). Similarly, the expression of arginase I is increased in ischemic myocardium (66). Increased arginase activity has also been shown in patients with myocardial infarction and this change correlated with the area of necrosis (100, 101). The activity of arginase increased in infarcted myocardium compared to normal myocardium. Furthermore, an increased concentration of urea on the venous side compared to arterial was observed, suggesting a local production of urea by arginase (102). Other factors contributing to increase arginase activity is hypoxia (103), relevant in IR (66), angiotensin (104) and thrombin (90).

These observations suggest that arginase is up-regulated in settings of inflammation, hyperlipidemia, atherosclerosis, diabetes, and myocardial IR.

Functional role of arginase

Based on the experimental evidence demonstrating increased arginase activity and reduced NO bioavailability in several cardiovascular disease conditions, studies using arginase inhibitors have been performed to evaluate the functional role of arginase *in vivo*. Experimental studies have described improvements in endothelial function by inhibiting arginase in various experimental models of cardiovascular disease including atherosclerosis, hypertension and diabetes type 1 and type 2 (73). Arginase inhibition restored endothelial function in hypertensive rats (105, 106). NO production and endothelium-dependent vasodilatation was increased following silencing of arginase I in rat aortas (79). Another experiment evaluated endothelial function and vascular stiffness in old rats. It was shown that arginase activity was higher in old rats compare to young and that arginase inhibition improved endothelial function, vascular compliance, and reduced NOS uncoupling in the old rats (107). Rings

from rat aortas incubated with oxLDL decreased NO metabolites production and increased arginase activity. These changes were reversed by arginase inhibition (108). The same study also showed a time-dependent increase of arginase in human aortic endothelial cells exposed to oxLDL. Arginase II inhibition or deletion of the arginase II gene prevented the decreased in NO levels, ROS production, vascular stiffness, and restored endothelial function (91). Experimental evidence also suggest an important functional role of arginase in diabetes. Arginase inhibition reversed the impairment in endothelium-dependent vasodilation in coronary arteries in diabetic rats (78). Rats with diabetes had reduced coronary flow reserve compared to healthy rats *in vivo* (77). Arginase inhibition restored coronary flow reserve in diabetic animals to levels seen in healthy animals (77). Furthermore, expression and amino acid analyses suggested that arginase is up-regulated in diabetic animals (77). Together, this suggests that arginase is of importance for microvascular dysfunction in rats with diabetes. Furthermore, the positive effect of arginase inhibition was abolished by co-administration of a NOS-inhibitor, suggesting the effect of arginase inhibition is mediated by NOS (77). In the same paper arginase II expression was increased in aorta and myocardium in rats with diabetes, and the citrulline/ornithine ratios, reflecting the indirect measurement of NOS versus arginase activity, increased more in the diabetes group after intervention. These observations offer further support to the idea that arginase is of importance for vascular homeostasis.

Even though there are multiple studies evaluating the acute effect of arginase inhibition the long-term effects of this intervention is largely unknown. One study exposed spontaneously hypertensive rats to ten weeks of arginase inhibition which resulted in antihypertensive effect and prevention of remodeling of the aorta and myocardial fibrosis without any reported side effects (109). This indicates that arginase inhibition could have effect on long-term remodeling, which could be positive in the treatment of cardiovascular disease.

Data from clinical studies on patients with cardiovascular disease are sparse. These are limited to *ex vivo* studies on coronary arterioles obtained from patients with diabetes (98) and *in vivo* where subcutaneous administration by microdialysis of arginase inhibitor to patients with hypertension showed attenuation of cutaneous vasodilatation in hypertensive subjects (110). In contrast, L-arginine supplementation failed to generate a positive effect on this outcome. The same investigator showed that arginase inhibition administered by microdialysis improved cutaneous vasodilatation in old humans compared to young individuals (111). These studies suggest that arginase inhibition improves vascular function under these conditions.

Since arginase is upregulated, and NO bioavailability is reduced during myocardial IR, previous studies have investigated the pathophysiological role of arginase in the setting of myocardial IR. In a rat model of 30 minutes coronary artery ligation followed by two hours reperfusion it was shown that administration of the arginase inhibitor N^{ω}-hydroxy-L-arginine (nor-NOHA) 15 minutes before ischemia reduced infarct size by approximately 50% (66). This cardioprotective effect was abolished after co-administration of the NOS-inhibitor L-N^{G}-monomethyl arginine (L-NMMA) suggesting the effects of arginase inhibition is mediated by NOS (66, 77). Diabetic and non-diabetic rats demonstrated reduced infarct size after administration of arginase inhibition in an experimental model of myocardial infarction (112). This is of importance considering the up-regulation of arginase in diabetes and in IR (113), the poor outcome of patients with diabetes suffering from myocardial infarction (60), and the unfavorable outcome after revascularization among patients with diabetes (114, 115).

Diabetes contributes to an unfavorable milieu after IR by promoting endothelial dysfunction, alteration of neutrophil infiltration, and reduced eNOS function (114). Hence, it is of great importance to evaluate arginase inhibition in the setting of IR in subjects with diabetes.

Collectively, available data suggest that arginase plays and important role in cardiovascular disease and that targeting arginase activity may be a promising novel therapeutic strategy. However, no *in vivo* data is available regarding the efficacy of arginase inhibition to improve endothelial function in patients with risk factors such as diabetes or clinically apparent CAD. Based on the above mentioned experimental data arginase inhibition could be of clinical benefit in the condition of diabetes, hypercholesterolemia, or IR.

Treatments restoring the bioavailability NO have shown promising results in experimental studies. However, many of the therapies have failed to prove successful in the clinical setting. In addition, traditional intensive glucose lowering therapy does not seem to decrease the mortality from cardiovascular disease in patients with diabetes, suggesting new mechanisms should be explored. Furthermore, today there are no drugs specifically targeting the molecular mechanisms behind endothelial dysfunction in patients with diabetes, CAD, or hypercholesterolemia. This thesis will evaluate the functional significance of inhibiting arginase for endothelial function in patients with glucose and lipid abnormalities.

Objective and Aims

The objective was to test the hypothesis that the enzyme arginase plays a key role in the development of micro- and macrovascular dysfunction in patients with glucometabolic disorders by reducing the bioavailability of NO. This hypothesis was tested in interventional studies using arginase inhibition in patients with type 2 diabetes and hypercholesterolemia with and without clinically apparent cardiovascular disease.

The specific aims were:

1. To investigate the effect of arginase inhibition on peripheral endothelial function in patients with CAD and type 2 diabetes.

2. To investigate the therapeutic effect of arginase inhibition on microvascular function in type 2 diabetes.

3. To evaluate the effect of arginase inhibition on IR-induced endothelial dysfunction in CAD and type 2 diabetes.

4. To investigate the regulatory role of arginase on endothelial function in patients with hypercholesterolemia.

METHODS

Study subjects

All study subjects were recruited from Karolinska University Hospital. These studies were conducted according to the declaration of Helsinki and approved by the local ethical committee. Study subjects were informed about the possible harm and all subjects gave their oral and written consent. All subjects tolerated the study protocols well.

General exclusion criteria were a) participation in another ongoing study b) unwillingness to participate c) myocardial infarction or instable angina within three months d) disease or condition that reduced the patients' ability to complete the study protocol e) arterio-venous shunts f) other vascular anomalies, such as Raynauds phenomenon g) age > 80 years h) ongoing treatment with oral anticoagulants.

Study I
In **Study I**, 16 patients with CAD, defined as 50% stenosis on coronary angiography or previous medical history of myocardial infarction, were included. In addition, 16 patients with both CAD and diabetes were included. Diabetes was defined as two occasions of elevated fasting glucose of above 7.0 mmol/l or levels exceeding 11.0 mmol/l 2 hours after 75 g of an oral glucose load. All patients were recruited from the Department of Cardiology at Karolinska University Hospital. The control group consisted of age-matched subjects without medications and history of cardiovascular disease. The control group was screened by an exercise tolerance test and an oral glucose tolerance test.

Study II
Patients were recruited from the Department of Endocrinology, Metabolism and Diabetology at Karolinska University Hospital. Patients with diabetes and microvascular dysfunction were included. Diabetes was defined as above and microvascular dysfunction was defined as either retinopathy or microalbuminuria > 3.0 mg/mmol. Furthermore, a control group of 12 healthy age-match controls was recruited.

Study III
Patients were recruited from the Department of Cardiology at Karolinska University Hospital. Twelve subjects with CAD and 12 subjects with both CAD and diabetes were included. Inclusion criteria were as in **Study I**.

Study IV
Twelve patients with FH were recruited from the Department of Endocrinology, Metabolism and Diabetology or the Department of Cardiology at Karolinska University Hospital. An age-matched control group without medications, history of cardiovascular disease, or history of diabetes was recruited. The FH diagnosis was determined by the Dutch Lipid Clinic Network criteria (116). Exclusion criteria were clinical symptom or history of cardiovascular disease, hypertension, diabetes, excessive alcohol consumption, or microalbuminuria.

Measurement of endothelial function

In general, all techniques for evaluating endothelial function consist of two parts 1) a technique to measure blood flow or vessel diameter and 2) stimulation of the endothelium by pharmacologic agents (serotonin or acetylcholine) or by increased shear stress. This increase in blood flow or vessel diameter in response to an endothelium-specific stimulus is referred to as endothelium-dependent vasodilatation (EDV) and is the result of NO produced by the endothelium. Endothelium-independent vasodilatation (EIDV) is the increase in blood flow during administration of an NO donor which acts on the smooth muscle cells directly without involving the endothelium.

Venous occlusion plethysmography

Venous occlusion plethysmography is a method for evaluating the arterial inflow into the forearm. It was used in **Study I and IV**. The blood flow was measured by placing a strain-gauge (a device measuring the change in circumference) around the widest part of forearm. The change in circumference results in a change of volume which is related to arterial flow rate. For this measurement set-up, two blood pressure cuffs were placed on each arm, one at the level of the upper arm and one at the level of the wrist. The proximal cuff was inflated to 50 mmHg to stop venous blood from exiting the arm but allowing entry of arterial blood. The cuff at the wrist was inflated to 30 mmHg above systolic pressure to exclude the complex circulation of the hand. The proximal cuff was inflated in cycles of ten seconds during which the arm circumference increased (due to arterial blood entering the arm) followed by release of the cuff for five seconds during which the circumference returned to baseline. This procedure was performed eight times during each infusion of either saline, serotonin, or sodium-nitroprusside (SNP) (Figure 5). The clinical use of this method originally was to evaluate a clinical diagnosis of venous thrombosis. Over time, great knowledge of human physiology has been shared among clinicians in many generations (117).

Flow mediated vasodilatation

Flow mediated vasodilatation (FMD) is a method based on ultrasound, using a high frequency (11 MHz) linear ultrasound probe placed over an artery to record the diameter of the vessel, usually the radial or brachial artery. The diameter of the vessel is recorded before and after a period of 5 minutes of local ischemia to the arm using a blood pressure cuff placed on the forearm and inflated to 30 mmHg above systolic pressure. The hyperemia occurring following deflation of the cuff increases the sheer stress to the endothelium. Shear stress stimulates the endothelium to produce NO, which mediates dilatation of the artery detected by ultrasound and is a measurement of endothelial function (Figure 6). The diameters are recorded in the same time point in each cardiac cycle to generate comparable data. This method was used in **Study III**, in which we evaluated the radial artery in the non-dominant arm using an 11 MHz transducer connected to a Vivid E9®. Images were recorded every third second at end diastole. A tripod was used to minimize any movement artefact. Baseline measurement was recorded as the mean of 20 frames. The effect size for the experiment was a ratio of the mean diameter of the three highest values after ischemia divided by the mean diameter recorded at baseline.

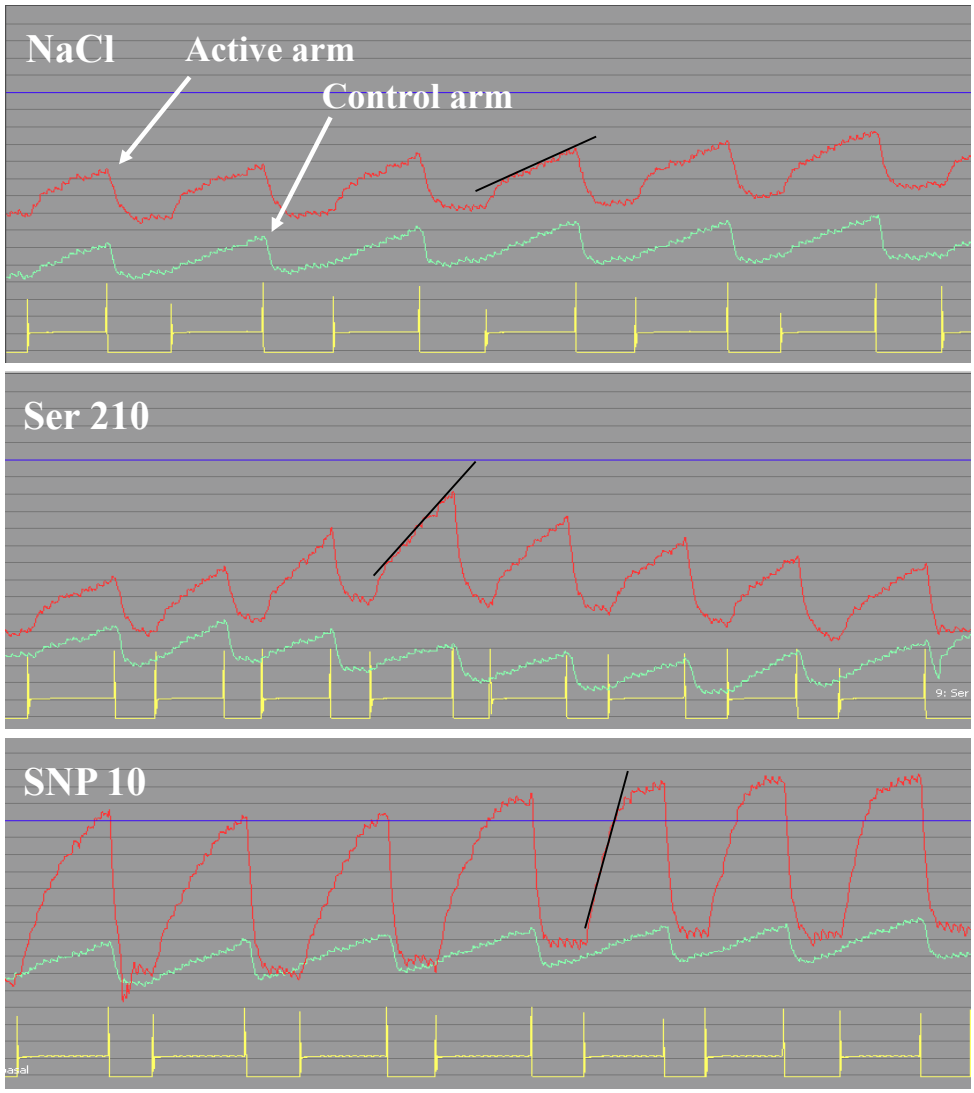

Figure 5. Recordings from investigation with venous occlusion plethysmography. The circumference of the forearm is recorded in active (red curves) and control arm (green curves). The circumference increases when the blood pressure cuffs are inflated (yellow line). By placing a tangent in lining with the increase in diameter (black tangent in the middle figure) in the four steepest curves, forearm blood flow was calculated. A set of eight curves were recorded over a period of 2 minutes. Baseline forearm blood flow is evaluated during saline administration (top figure). Endothelium-dependent vasodilatation is stimulated with serotonin. The middle panel demonstrates the response in forearm blood flow to the highest concentration of serotonin. Endothelium-independent vasodilatation is stimulated with sodium-nitroprusside (bottom). NaCl, saline; Ser 210, serotonin 210 ng/min; SNP 10, sodium-nitroprusside 10 µg/min.

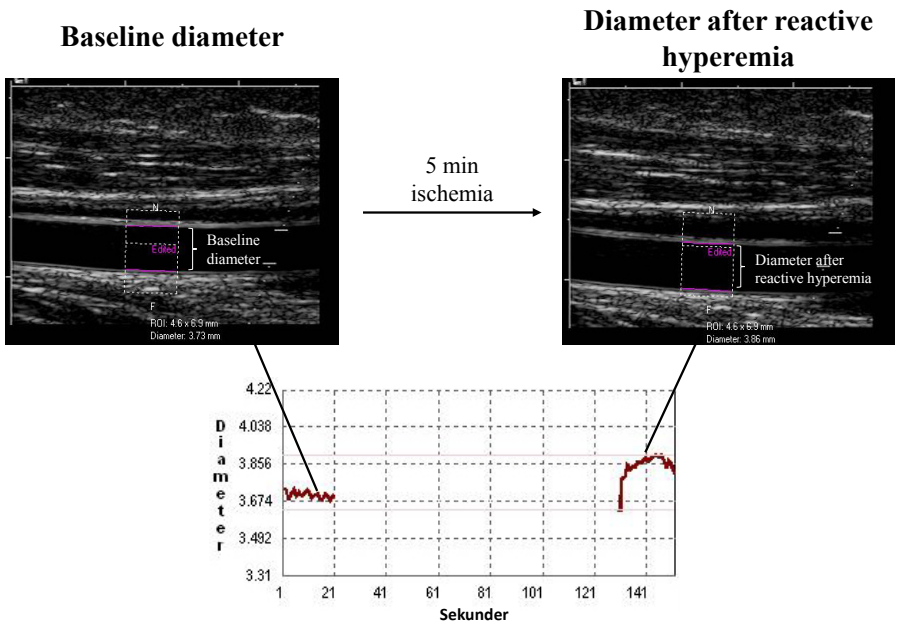

Figure 6. Flow-mediated vasodilatation recordings. Baseline artery diameter is recorded with an automated edge detection (picture top left). After 5 minutes local ischemia, reactive hyperemia is induced by decreased resistance after ischemia, new frames were recorded (top right). The dilatation is visualized in the lower figure where baseline and maximal artery diameters are presented. Frames are recorded at end-diastole every third second during the period of measurement. FMD; flow-mediated vasodilatation.

Laser Doppler flowmetry and iontophoresis

Laser Doppler flowmetry (LDF) is, in essence, a laser beam that projects onto the skin and is reflected by red blood cells within the cutaneous microcirculation. This method is used to estimate indirectly blood flow in the microvasculature. As the technique just reflects the amount of red blood cells at one point in the skin, it is not possible to estimate actual blood flow and instead an arbitrary measure of flow is obtained. The probes containing the laser Doppler also have the ability to deliver substances locally to the skin. By doing so it is possible to investigate the EDV by administering acetylcholine and the EIDV with SNP within the microcirculation. Acetylcholine and SNP are delivered into the skin using iontophoresis i.e. by using an electrical current. This method was used in **Study II**. Two probes were placed on the subjects arm 5 cm apart. After the baseline flow had been recorded for 3 minutes, acetylcholine and SNP were administered simultaneously using currents of 0.08, 0.14, and 0.20 mA. Each of the three stimulations was maintained for one minute and then recorded for 15 min to ensure the maximum response for each substance.

Substances

N^{ω}-hydroxy-nor-L-arginine (Nor-NOHA) (Bachem, Bubendorf, Switzerland) and serotonin (Sigma-Aldrich, Schnelldorf, Germany), were dissolved in double-distilled water sterile filtered through a Milipore filter, tested for bacteria and toxins and frozen at – 80°C.

Sodium nitroprusside (SNP, Abbot, Chicago) and L-N-monomethyl L-arginine (L-NMMA) (Clinalfa, Läufelfingen, Switerland) were diluted in 0.9 % saline to proper concentration on the day of the experiment.

Acetylcholine (Bausch & Lomb Nordic AB, Stockholm, Sweden) was diluted in 0.9% saline.

Nitroglycerine (0.4 mg, PharmaPol, Dägeling, Germany).

Local anesthesia using Xylocaine 10 mg/ml (AstraZeneca, Sweden).

Study protocols

Blood samples were collected in the fasting state at the beginning of each protocol.

Study I

Forearm blood flow (FBF) was recorded by venous occlusion plethysmography in both arms simultaneously. Following infiltration with local anesthesia, a catheter was introduced in the brachial artery of the non-dominant arm for i.a. infusions. A venous catheter was introduced in a deep cubital vein of the same arm to collect blood perfusing the forearm. Baseline FBF was recorded during a saline infusion of 3.5 ml/min. EDV and EIDV were recorded during infusion of 21, 70, and 210 ng/min for serotonin and 1, 3, and 10 µg/min for SNP at a rate of 2.5 ml/min together with 1 ml/min saline. After baseline EDV and EIDV were recorded, administration of nor-NOHA was started instead of saline and given at a rate of 0.1 mg/min (1 ml/min). Nor-NOHA was administered for 120 min, after which basal FBF, EDV, EIDV were evaluated again in the same manner. For a visual representation of the protocol, see Figure 7. Five patients with CAD and diabetes participated in one additional protocol. To examine if the effect of nor-NOHA was dependent on NOS, the NOS-inhibitor L-NMMA was co-administered with nor-NOHA at a rate of 2 mg/min (1 ml/min).

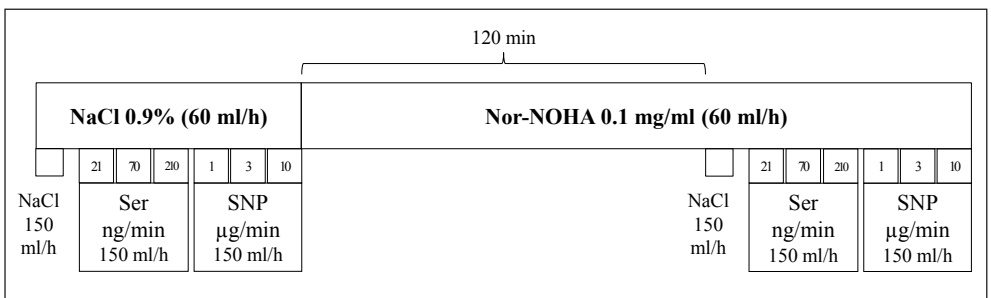

Figure 7. The protocol of **Study I**. During the administration of i.a saline forearm blood flow was recorded during co-administration of serotonin (21, 70, and 210 ng/min) and sodium-nitroprusside (1, 3, and 10 µg/min). Each infusion was maintained for 2 minutes. The same parameters were evaluated after 120 minutes i.a. administration of nor-NOHA (0.1 mg/min). NaCl, saline; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine; Ser, serotonin; SNP, sodium-nitroprusside.

Study II

Microvascular endothelial function was measured by LDF. Two probes containing drug delivery system and laser Doppler were placed on the subjects' non-dominant forearm 5 cm apart. Shallow veins, scars and discolorations was avoided. An arterial catheter was introduced in the brachial artery to administer saline and the arginase inhibitor nor-NOHA (0.1 mg/min). Acetylcholine and SNP to stimulate EDV and EIDV were administered simultaneously by iontophoresis using currents of 0.08, 0.14, and 0.20 mA each stimulus was maintained for 60 seconds. The microvascular response to these stimuli was recorded for 15 min after each stimulation. The evaluation of EDV and EIDV were evaluated before and after 120 min of nor-NOHA administration (Figure 8).

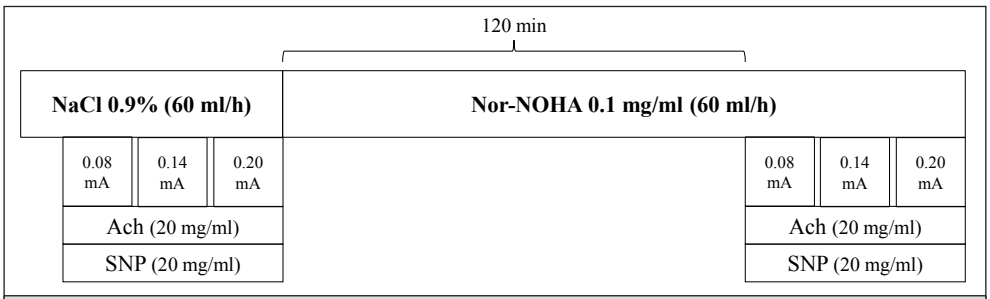

Figure 8. The protocol of **Study II**. Laser Doppler flowmetry was measured during transcutaneous iontophoretic delivery of acetylcholine and sodium-nitroprusside (SNP) in three doses was administered during saline administration and again after 120 minutes of i.a. administration of nor-NOHA. Each stimulation was maintained for 60 seconds and recorded during 15 minutes. The concentration of acetylcholine and SNP was 20 mg/ml. Ach, acetylcholine; NaCl, saline; mA, milliampere; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine; SNP, sodium-nitroprusside.

Study III

Subjects with CAD with and without diabetes were subjected to two different protocols. Catheters were introduced into the brachial artery and a deep vein catheter of the non-dominant arm. A vacuum cushion was used to reduce the movement of the arm. EDV was measured by FMD (see above) at baseline and after 40 minutes of IR, i.e. 20 minutes of ischemia and 20 minutes of reperfusion. During the two protocols, 0.9% NaCl or nor-NOHA (0.1 mg/min) were given as i.a. infusions at a rate of 1 ml/min. The infusions started 15 minutes into the period of ischemia and were maintained for 20 minutes (Figure 9). The order of the protocols was randomized by blindly drawing one out of two numbers printed on two different pieces of paper.

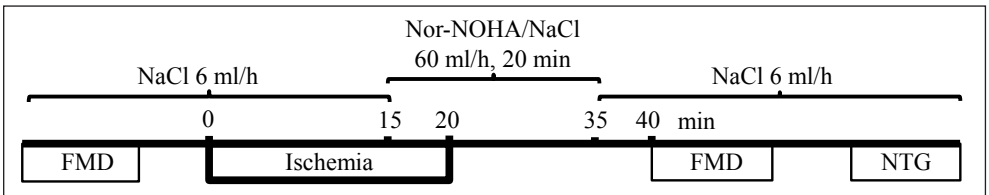

Figure 9. The protocol of **Study III**. FMD was evaluated at baseline and after 20 minutes of ischemia and 20 minutes of reperfusion. Either saline or nor-NOHA was given by i.a. infusions. All subjects performed the protocol twice. FMD; flow-mediated vasodilatation, nor-NOHA; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine, NTG; nitroglycerine.

Study IV
In **Study IV**, patients with FH performed the protocol twice. During the first visit, FH subjects had stable medication with lipid-lowering drugs. All subjects were prescribed statins (n=12) and two had additional treatment with ezetimibe. After visit 1 all lipid-lowering drugs were discontinued for four weeks, after which the second visit was performed. Control subjects were evaluated once. The protocol was similar to that of **Study I** with the exception that the doses of serotonin used to evaluate EDV were reduced on the basis of a younger and healthier patient group and one extra dose was added. The doses of serotonin that were used were 5.25, 17.5, 52.5, and 175 ng/min. EIDV was evaluated by administration of SNP as in **Study I**. FBF, EDV, and EIDV were evaluated before and after 120 minutes of nor-NOHA administration (0.1 mg/min).

Analysis of arginase expression and amino acids
Arginase expression was analyzed by immunohistochemistry. A small section of the left internal mammary artery was harvested from patients with stable CAD with and without diabetes undergoing coronary artery bypass grafting surgery. The distal end of the artery was sliced and frozen in dry ice. Samples were sectioned, fixed with acetone, and stained with MACH 3 technology (Biocare Medical). Primary antibodies against arginase I, arginase II, alpha-actin, and von Willebrand factor were incubated together with the sections for one hour at room temperature.

For determination of the amino acids L-arginine, ornithine, and citrulline, liquid chromatography tandem mass spectrometry was used (118).

Statistical analysis

In all studies, statistical analyses were performed with GraphPad Prism (Version 5.0/6.0 GraphPad software Inc, La Jolla, CA, USA).

Study I
We estimated that 12 subjects were sufficient to detect a significant improvement in EDV.

Study II
Based on a previous interventional study of microvascular function of comparable design (119) we estimated that 12 individuals were sufficient to detect a significant improvement in endothelium-dependent dilatation.

Study III
Based on an absolute improvement of FMD of 3% and a SD of 1.85%, the number of subjects needed to reach 80% power with a 2-sided test with a significance level of 5% was calculated to be 12.

Study IV
In **Study I**, we observed an improvement in EDV by a mean of 18 ml/min/1000 ml in patients with coronary artery disease and type 2 diabetes. Assuming an improvement of 12 ml/min/1000 ml in the current population, we calculated that 11 individuals would be sufficient to reach 80% power with a 2-sided test with a significance level of 5%.

The data from subject characteristics was normally distributed and was analyzed with Student's t-test when comparing two groups. In the case of inclusion of three groups, as in **Study I**, unpaired one-way analysis of variance was used.

In EDV and EIDV data contained repeated measurements before and after intervention of each subject and all studies included at least two groups. Comparison between baseline and after intervention within each group was performed using paired analysis whereas unpaired analysis was used for comparisons between groups. Two-way analysis of variance with repeated measurements was used to analyze the data. Basal blood flow (**Studies I and IV**) and radial artery diameter (**Study III**) were analyzed with one-way analysis of variance.

Normal distribution of data was determined using the D'Agostino-Pearson normality test and plotted for visual evaluation.

RESULTS

For patient characteristics and medications, please see tables 1 and 2 respectively.

Study I

Patients with CAD and diabetes had significantly higher glucose and HbA1c levels than the other groups. No difference was observed regarding blood pressure. The patient groups had lower levels of cholesterol than the control group since they were on lipid-lowering medication (Tables 1 and 2).

To determine the expression of arginase isoforms we stained for arginase I and II in the left internal mammary artery obtained from CAD patients with and without diabetes using immunohistochemistry. Both isoforms of arginase were expressed but at different locations in the vessel wall. Arginase I was expressed both in endothelial cells and in smooth muscle cells, whereas arginase II was expressed more selectively in endothelial cells (Figure 10). With the knowledge that arginase was expressed in arteries of patients with CAD with and without diabetes we proceeded with studies evaluating the functional importance of arginase. There were no significant differences in FBF at baseline between the groups. Further, administration of nor-NOHA did not affect baseline FBF in any of the groups. Patients with CAD had a reduced baseline EDV compared to healthy subjects (Figure 11, $P<0.01$). Patients with both CAD and diabetes had worse baseline EDV than patients with CAD alone (Figure 11, $P<0.01$). Baseline EIDV was reduced in patients with CAD ($P<0.05$) and CAD and diabetes ($P<0.01$) compared to healthy subjects.

The role of arginase in regulation of endothelial function was tested by the administration of the arginase inhibitor nor-NOHA. This induced a significant increase in EDV in subjects with CAD and diabetes as well as in patients with CAD alone (Figure 11). By contrast, no increase in EDV was seen among healthy subjects after administration of nor-NOHA. Importantly, the improvement in EDV after nor-NOHA administration was greater in the CAD and diabetes group than in the CAD group (Figure 12). EIDV increased slightly in the CAD and diabetes group ($P<0.001$) following nor-NOHA administration, but it remained unchanged in the other two groups.

We investigated further whether NOS was involved in the improvement in EDV induced by arginase inhibition. Interestingly, the NOS inhibitor L-NMMA completely abolished the positive effect of nor-NOHA on EDV (Figure 13), suggesting that the effect of arginase inhibition was mediated via a NOS-dependent pathway.

Study II

In light of our findings from **Study I**, i.e. that subjects with diabetes had a significantly greater improvement in endothelial function in response to arginase inhibition, we investigated if arginase was of importance for microvascular dysfunction in subjects with diabetes.

The diabetes group had higher BMI, C-reactive protein, fasting glucose and HbA1c, but lower total, LDL and HDL cholesterol compared to the control group (Table 1). None of the subjects were smokers. Subjects in the control group did not take any medication. The medications of the groups are listed in Table 2.

Table 1. Basal characteristics of subjects included in this thesis. Data are presented as mean ± SD. Differences in basal characteristics were analyzed within each study. **Study I**, *P<0.05 vs. the control group, **P<0.01 vs. the control group, ***P<0.001 vs. the control group, ###P<0.001 vs. the CAD group. In **Study II**, **P<0.01 vs. the control group, ***P<0.001 vs. the control group. In **Study III**, **P<0.01 vs. the CAD group, ***P<0.001 vs. the CAD group. In **Study IV**, *P < 0.05; **P < 0.01; ***P < 0.001, compared to visit 1. †††P < 0.001, compared to visit 2. BMI, body-mass index; CAD, coronary artery disease; FH, familial hypercholesterolemia; HDL, high-density lipoprotein; HbA1c, glycosylated hemoglobin; LDL, low-density lipoprotein; MAP, mean arterial pressure.

	Study I			**Study II**		**Study III**		**Study IV**		
	Controls (n=16)	CAD (n=16)	CAD+diabetes (n=16)	Controls (n=12)	Diabetes (n=12)	CAD (n=12)	CAD+diabetes (n=12)	Controls (n=12)	FH (n=12)	
									Visit	
									1st	2nd
Age (y)	61± 5	62 ± 6	64 ± 10	66±4	66±6	65 ± 8	65 ± 7	30±7	32±10	32±10
Blood pressure (mmHg)										
systolic	125±9	133±20	136±15	136±16	145±21	131±15	141±12	124±12	119±8	118±6
diastolic	78±8	78±7	75±12	81±7	76±14	80±9	83±10	74±11	76±7	75±7
BMI (kg/m^2)	26 ± 3	30±11	29±4	24±1	34±11**	28±4	27±3	23.5±3.3	25.7±4.1	nd
Waist-hip ratio	0.94±0.03	0.97±0.06	0.99±0.03**	0.98±0.06	1.02±0.06	0.99±0.06	0.98±0.06	0.94±0.06	0.89±0.07	nd
Fasting glucose (mmol/L)	5.2±0.4	5.4±0.7	8.1±2.2***,###	5.6±0.4	9.7±3.5***	5.6±0.8	8.0 ± 2.2 **	5.4±0.8	5.2±0.2	5.0±0.3
HbA1c (%)	38±3	38 ±4	52±10***,###	36±2	66±14***	39 ± 3	52 ± 8 ***	n/a	n/a	n/a
Triglycerides (mmol/L)	1.1±0.8	1.2±0.6	2.1 ±1.5*	1.1±0.6	1.3±0.8	1.3±0.8	1.9±1.0	0.96±0.47	0.90±0.41	1.39±0.73*
Total cholesterol (mmol/L)	5.6±0.8	4.1±0.7***	3.8±0.9***	5.8±1.2	3.2±0.7***	4.3±0.8	4.2±1.1	4.2±1.0**,†††	6.0±1.0	9.5±2.0***
LDL (mmol/L)	3.7±0.7	2.4±0.6***	2.0±0.9***	3.6±0.9	1.5±0.5***	2.4±0.5	2.2±0.8	2.4±0.9***,†††	4.3±0.9	7.6±1.9***
HDL (mmol/L)	1.4±0.3	1.2±0.2	1.1±0.4*	1.7±0.4	1.2±0.3**	1.3±0.3	1.1±0.3	1.3±0.3	1.3±0.3	1.2±0.3
Creatinine (µmol/L)	79±26	82±15	92±50	81±7	107±43	82±14	92±54	83±10	79±10	75±10

Table 2. Medication taken by subjects included in this thesis. Data are presented as absolute numbers. ACE inhibitors, angiotensin converting enzyme antagonists; ARB, angiotensin II receptor blocker; CAD, coronary artery disease; FH, familial hypercholesterolemia.

	Study I			**Study II**		**Study III**		**Study IV**		
	Controls (n=16)	CAD (n=16)	CAD+diabetes (n=16)	Controls (n=12)	Diabetes (n=12)	CAD (n=12)	CAD+diabetes (n=12)	Controls (n=12)	FH (n=12)	
									Visit	
Medication (no.)									1st	2nd
Lipid-lowering drugs	0	12	15	0	9	11	10	0	12	0
ACE-inhibitors or ARB	0	9	8	0	10	8	10			
Beta-blockers	0	14	12	0	5	11	8			
Antiplatelet drugs	0	15	15	0	12	12	12			
Calcium channel blockers	0	0	5	0	5	0	3			
Diuretics	0	1	4	0	3	2	3			
Nitrates	0	3	3	0	2	1	4			
Oral glucose lowering drugs	0	0	9	0	7	0	8			
Insulin	0	0	5	0	10	0	4			

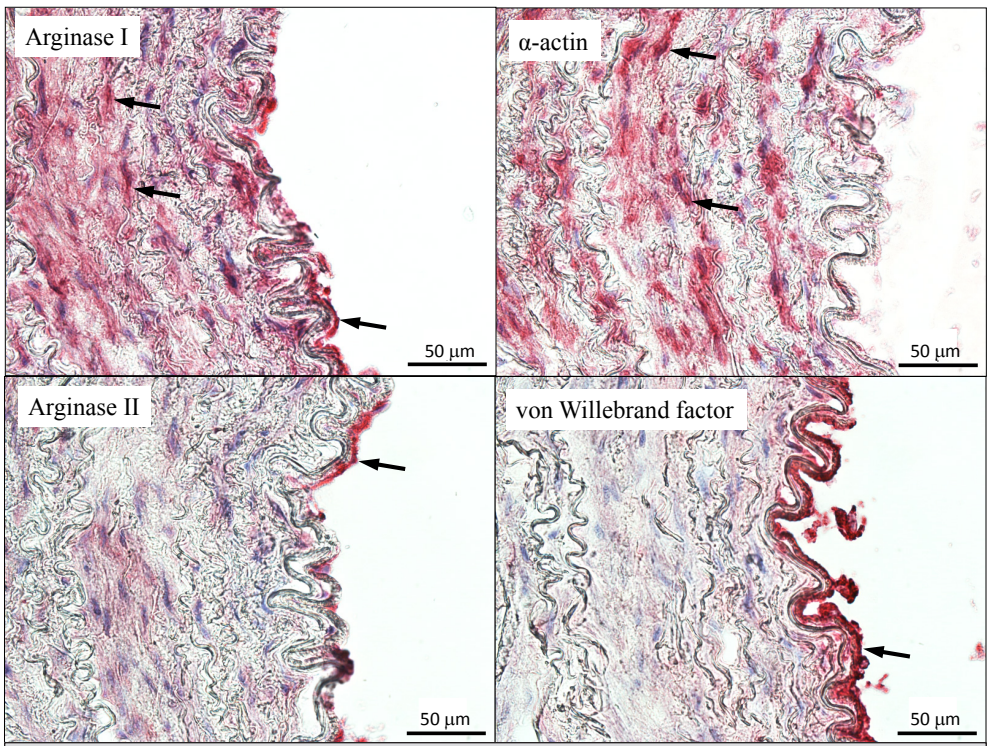

Figure 10. Immunohistochemical staining of left internal mammary artery from patient with CAD. Arginase expression is indicated in the figures to the left with black arrows. Expression of α-actin is used as marker for smooth muscle cells and von Willebrand factor for endothelial cells. Arginase I is present in both the endothelium and in the smooth muscle cells whereas arginase II is predominately expressed in the endothelium.

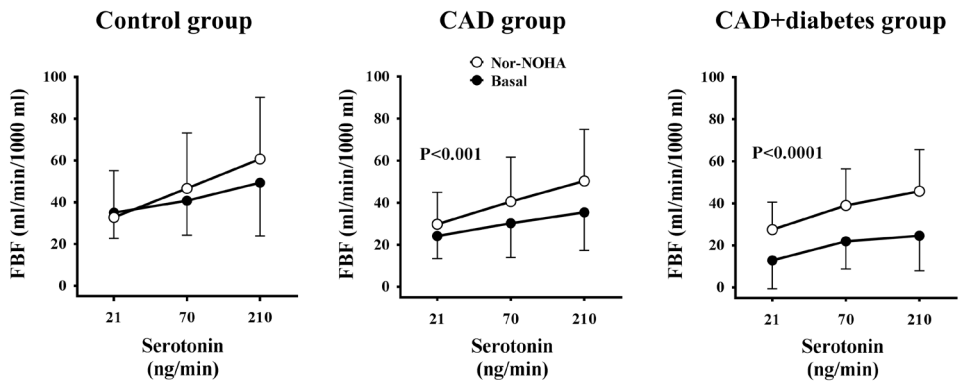

Figure 11. Results of endothelium-dependent vasodilatation (EDV) from **Study I**. EDV was not effected in the control group after arginase inhibition. However, arginase inhibition improved EDV in CAD subjects both with and without diabetes. Data is presented as mean ± SD. Significant differences between EDV following nor-NOHA in comparison with baseline EDV are presented. CAD, coronary artery disease; FBF, forearm blood flow; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine.

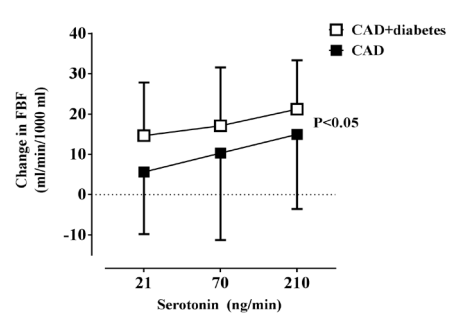

Figure 12. Change in EDV in response to arginase inhibition in patients with CAD and CAD and diabetes. The change was calculated as the difference in EDV between EDV during arginase inhibition and EDV at baseline. The improvement was greater in the CAD and diabetes group compared to patients with CAD only. Data are presented as mean ± SD. CAD, coronary artery disease; FBF, forearm blood flow; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine.

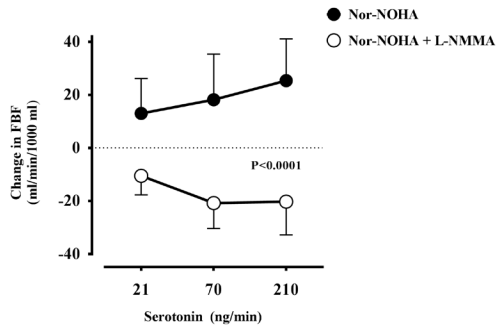

Figure 13. To investigate the mechanism behind arginase inhibition patients with CAD and diabetes performed one additional protocol in which the NOS-inhibitor L-NNMA was administered. The change was calculated as the difference in EDV between EDV during arginase inhibition and EDV at baseline. The positive change in EDV was abolished after NOS-inhibition, suggesting that the improvement in EDV is NOS-dependent. Data are presented as mean ± SD. CAD, coronary artery disease; FBF, forearm blood flow; L-NMMA, L-NG-monomethyl arginine; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine.

To evaluate if basal microvascular endothelial function was reduced in subjects with diabetes we recorded the EDV response during saline administration. Baseline microvascular EDV (increase in flow evoked by acetylcholine) was reduced in patients with diabetes compared to healthy subjects (Figure 14). Interestingly, after nor-NOHA administration, EDV increased in the diabetes group ($P<0.05$) to the level observed in healthy subjects (Figure 14). EDV in the control group did not change in response to arginase inhibition. EIDV (increase in flow evoked by SNP) did not differ between the groups nor did it change significantly within each group in response to arginase inhibition.

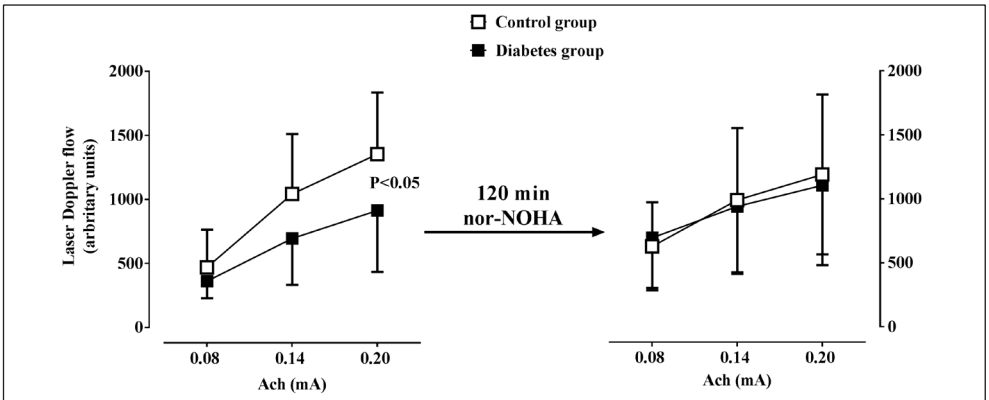

Figure 14. Microvascular endothelium-dependent and endothelium-independent function measured with laser Doppler flowmetry before and after 120 minutes i.a. administration of nor-NOHA. The diabetes group had reduced microvascular endothelial function at baseline compared to the control subjects ($P<0.05$). Arginase inhibition improved microvascular endothelial function in the diabetes group to the level of control subjects after intervention. Data are presented as mean ± SD. Ach, acetylcholine; mA, milliampere; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine; SNP, sodium-nitroprusside.

To quantify the relative activity between arginase and NOS, amino acid substrate and products of each enzyme were determined in plasma from healthy control subjects and patients with diabetes (Table 3). The arginase product ornithine was significantly higher among patients with diabetes than healthy subjects (71.3±28.1 vs. 50.0±8.2 µmol/L, $P<0.05$). The NOS product citrulline or the substrate L-arginine did not differ between the groups. Ratios was calculated to estimate the relative activity of NOS and arginase. Ornithine/L-arginine and ornithine/citrulline ratios were elevated in patients with diabetes compared to control subjects (0.91±0.28 vs. 0.65±0.16, $P<0.05$ and 1.99±0.73 vs. 1.41±0.38, respectively, $P<0.05$), suggesting an increased arginase activity in subjects with diabetes.

Table 3. Amino acid levels and ratios in **Study II**. Data are mean values ± SD. Significant differences between groups are shown: *$P<0.05$ vs. control subjects.

Amino acid (mmol/l)	Controls (n=12)	Type 2 diabetes (n=12)
Arginine	78.8 ± 14.4	79.2 ± 18.0
Ornithine	50.0 ± 8.2	71.3 ± 28.1*
Citrulline	37.9 ± 10.7	38.4 ± 13.7
Ratios		
Ornithine/Arginine	0.65 ± 0.16	0.91 ± 0.28*
Ornithine/Citrulline	1.41 ± 0.38	1.99 ± 0.73*
Citrulline/Arginine	0.49 ± 0.13	0.49 ± 0.18

Study III

As mentioned above, up-regulation of arginase in may reduce NO bioavailability contributing to endothelial dysfunction and IR injury. Therefore, the aim of this study was to investigate if arginase inhibition protects from endothelial dysfunction induced by IR in patients with CAD. Furthermore, since arginase seemed to be of particular importance for endothelial dysfunction in patients with diabetes (**Study I**), a group of subjects with CAD+diabetes was included.

Patients with CAD had lower fasting glucose and HbA1c than patients with both CAD+diabetes (Table 1).

FMD, i.e. the endothelium-dependent increase in artery diameter in response to increased shear stress, was reduced after IR compared to baseline in patients with CAD (Figure 15). Administration of nor-NOHA completely prevented the development of IR-induced reduction in FMD (Figure 15). Importantly, FMD in the CAD group after IR was significantly greater following administration of nor-NOHA than following administration of saline (Figure 15). Baseline FMD was impaired in the CAD+diabetes group compared to the CAD group (8.3±2.0% vs. 12.7±5.2%, $P<0.05$). FMD did not significantly decrease in response to IR in patients with CAD+diabetes during NaCl administration. However, arginase inhibition improved FMD following IR compared to baseline FMD in the CAD+diabetes group (Figure 15).

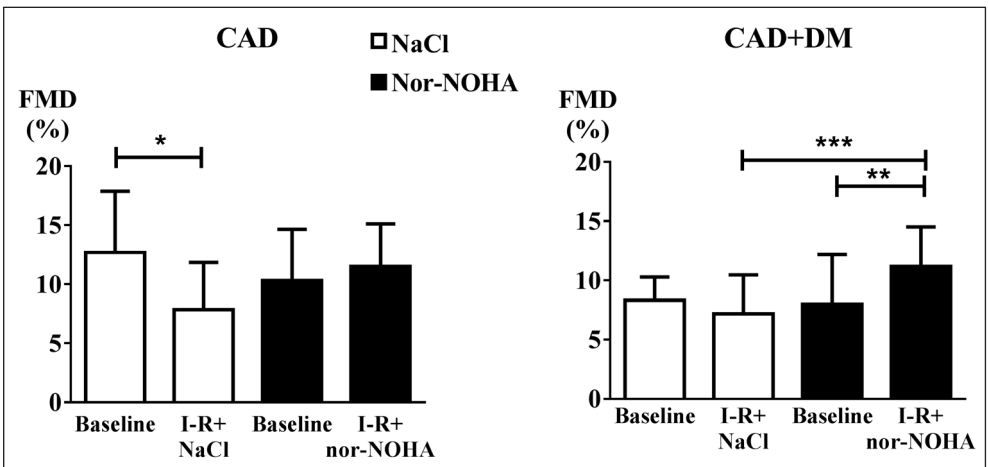

Figure 15. Flow-mediated vasodilatation (FMD) at baseline and after 20 minutes ischemia and 20 minutes reperfusion. Subjects with CAD decreased their FMD in response to ischemia-reperfusion (IR) during placebo administration ($P<0.05$). In contrast, nor-NOHA completely reversed this reduction. Baseline FMD was reduced among subjects with CAD+diabetes compared to CAD only ($P<0.05$). In the CAD and diabetes group FMD was not reduced in response to IR but nor-NOHA improved FMD above baseline despite IR. Data are presented as mean ± SD. I-R, ischemia-reperfusion; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

When all patients (CAD and CAD+diabetes) were analyzed together, FMD was reduced following IR and saline administration (10.5±4.4% baseline vs. 7.5±3.6% after IR, $P<0.01$) but FMD was not impaired after administration of nor-NOHA, instead a significant improvement in FMD was observed (9.2±4.3% baseline vs. 11.3±3.4% after IR, $P<0.05$).

EIDV induced by sublingual nitroglycerine did not differ between groups or between interventions. In the CAD group EIDV did not change between the two study visits (saline 11.8±4.1% vs. nor-NOHA 14.3±5.0%). The same was true for patients with CAD+diabetes (13.6±6.4% following saline and 12.3±6.7% following nor-NOHA administration). The baseline diameter of the radial artery did not change significantly neither before vs. after IR nor between the experiments in any of the groups.

Study IV

Recent experimental evidence has suggested a link between LDL and increased arginase activity, making hypercholesterolemia an important condition in which the functional significance of arginase needs to be explored.

The groups were well matched regarding all measured parameters except for cholesterol levels and medications (Tables 1 and 2). LDL cholesterol increased in subjects with FH after 4 weeks of abstaining from lipid lowering drugs compared to at inclusion (4.3±0.9 vs. 7.6±1.9 mmol/l, $P<0.001$). Despite their lipid-lowering medication, the FH subjects had higher LDL cholesterol at inclusion than the control subjects (2.4±0.9 mmol/l, $P<0.001$).

Baseline FBF did not differ between the groups nor did it change in response to nor-NOHA in any of the groups. Baseline EDV did not differ between the study visits or between the groups. Surprisingly, arginase inhibition increased EDV in all groups, including the control group. There were no changes in EDV in response to arginase inhibition between the first and second visit (low and high levels of LDL) of the FH patients. However, the improvement of EDV in response to nor-NOHA was greater among FH individuals than in healthy subjects (Figure 16). EIDV did not change in response to arginase inhibition nor did it differ between visits.

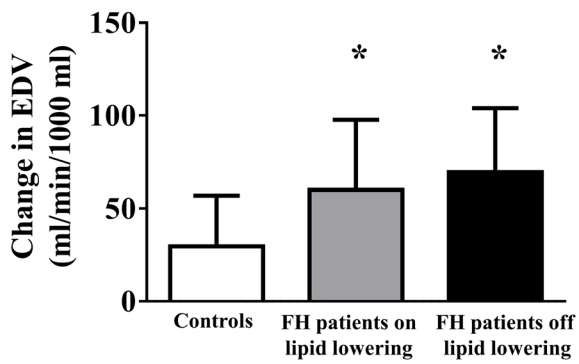

Figure 16. Improvement in endothelium-dependent vasodilatation after 120 minutes i.a. N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) administration from baseline in healthy controls, patients with familial hypercholesterolemia (FH) off lipid lowering therapy and FH patients on lipid lowering therapy. All groups responded to the intervention but the response in the subjects with FH was significantly greater than that of the control subjects. No difference was observed between the visits of subjects with FH. Data are presented as mean ± SD. *$P<0.05$ compared to the response in EDV from control subjects.

DISCUSSION

Key findings

The present studies demonstrate that arginase inhibition improves endothelial function in patients with CAD with and without diabetes, diabetes with microvascular dysfunction, and FH. The effect of arginase inhibition was evaluated in three different vascular beds *in vivo*.

The main findings are that:

- Arginase is expressed in left internal mammary arteries in patients with CAD and CAD+diabetes.
- Arginase inhibition improves endothelial function, via a NOS-dependent mechanism, among patients with CAD and especially among patients with CAD+diabetes.
- Arginase inhibition improves microvascular endothelial function in patients with diabetes and microvascular dysfunction.
- Amino acids reflecting relative arginase activity (in comparison to eNOS) is higher in patients with diabetes compared to control subjects.
- Arginase inhibition improves endothelial function in an IR model in patients with CAD, both with and without diabetes.
- Arginase inhibition improves endothelial function in FH, irrespective of LDL cholesterol levels.

The role of arginase in basal endothelial function in patients with CAD and diabetes

In **Study I**, the role of arginase inhibition on endothelial function was determined in subjects with CAD with and without diabetes and in healthy subjects. Patients with CAD showed reduced EDV compared to healthy controls, and subjects suffering from both CAD and diabetes presented further reduced EDV compared to patients with CAD only. These data confirm that the studied patient groups had different degrees of endothelial dysfunction at baseline. The level of endothelial dysfunction in the CAD and diabetes group is comparable to another study using the same methodology (117). In addition, impaired venous endothelial function was demonstrated *in vitro* in vessels from patients with CAD with and without diabetes compared to control subjects. In line with our results, the CAD and diabetes group had further impairment in endothelial function compared to the CAD group (120).

Arginase inhibition increased EDV in CAD patients with and without diabetes, but not in healthy subjects. Interestingly, the increase in EDV was significantly greater in the CAD and diabetes group compared to the group with CAD only, suggesting that arginase inhibition is more effective in subjects with CAD and diabetes. In line with our results, increased arginase I expression was demonstrated in coronary arteries from patients with diabetes (98). The same study observed reduced endothelial function in vessels from patients with and without

diabetes but endothelial function increased only in vessels from diabetic subjects after treatment with an arginase inhibitor. This provides further evidence that arginase inhibition can effect an improvement in endothelial function in the coronary arteries. As the positive effects of arginase inhibition were not observed in patients with CAD only, Beleznai and coworkers speculated that other mechanisms were behind the reduced endothelial function among patients without diabetes. However, our data obtained *in vivo* clearly suggest that that arginase is a key factor for endothelial dysfunction in CAD patients both with and without diabetes. This is also in line with our demonstration of expression of arginase I and II in endothelial cells of the left internal mammary artery from patients with CAD with as well as without diabetes.

Statins have been shown to reduce arginase activity and expression in an experimental model of diabetes (78). Furthermore, diabetic animals have reduced endothelial function which is completely reversed by treatment with statins, suggesting that statins could be used to improve endothelial dysfunction partly by inhibiting arginase (78). We demonstrate a clear improvement in endothelial function in both patient groups despite ongoing statin treatment, suggesting that arginase inhibition is of additional value in improving endothelial function beyond any effect of statins alone.

In **Study I** we observed a slightly increased response in EIDV in the CAD and diabetes group after arginase inhibition, indicating a possibility to improve the NO-mediated signaling in these individuals. The mechanism behind this observation is unclear but may be the increased degree of oxidative stress in subjects with diabetes (121). By inhibiting arginase, uncoupling of eNOS might be reduced which results in less ROS (107) to react with NO from the NO donor. This may possibly improve NO-signaling.

The effect of arginase inhibition in the microcirculation in patients with diabetes

Based on the clear improvement in endothelial function among subjects with diabetes described in **Study I**, we hypothesized that arginase could be an important regulator of endothelial function in the microvasculature. Hence, **Study II** sheds light on whether inhibition of arginase improves endothelial function in the microvasculature in subjects suffering from diabetes and microangiopathy.

Study II demonstrates a significant difference in basal microvascular endothelial function between the diabetes group and the control subjects. This provides evidence that the studied population actually has endothelial dysfunction that can be detected with the method of measurement used. This finding is to be expected since endothelial dysfunction is thought to be a key mechanism behind microangiopathy in diabetes (122, 123).

Arginase inhibition increased microvascular endothelium-dependent dilatation among subjects with diabetes but not in control subjects, similarly to **Study I**. Importantly, after arginase inhibition there was no significant difference in endothelial function between the groups, suggesting that arginase inhibition improves microvascular endothelial dysfunction to the level of healthy subjects in patients with diabetes. This effect was observed despite

the fact that the majority of patients were treated with stains and angiotensin converting enzyme inhibitors. Angiotensin converting enzyme inhibitors have been demonstrated to improve endothelial function in larger arteries and the coronary circulation (124), although, it seems that the effects of angiotensin converting enzyme inhibitors on endothelial function in resistance vessel and microcirculation are less clear (125). However, statins have been shown to improve microvascular dysfunction (126). Despite standard treatment, we observed impaired microvascular endothelial function among patients with diabetes and microangiopathy that could be reversed by arginase inhibition. This suggests that arginase inhibition further contribute to increasing endothelial function. In addition, 10 out of 12 patients with diabetes received at least one daily dose of insulin. Kashyap and colleagues present evidence that NOS activity was decreased 4-fold and that arginase activity in plasma was increased by 50% in a group of patients with diabetes compared to non-diabetic subjects (81). Furthermore, after insulin stimulation the level of arginase activity in plasma dropped in the diabetic subjects (81), suggesting a possible underestimation of the impact of arginase in these patients. In contrast to this finding we still observe additional improvement in microvascular endothelial function when inhibiting arginase in patients with diabetes. Five earlier studies have evaluated microvascular response to arginase inhibition in humans, although never in patients with diabetes (110, 111, 127-129). The results have been consistent, showing that arginase inhibition improves microvascular perfusion and endothelial function in examined patients. These findings further strengthen the idea that arginase is an important mediator of microvascular dysfunction. We provide evidence that inhibition of arginase improves microvascular endothelial dysfunction in patients with diabetes and microangiopathy.

Analysis of arginase activity *in vivo*

An additional observation of **Study II** was the analysis of amino acids associated with NOS and arginase metabolism. We show increase levels of ornithine (the product of arginase) in subjects with diabetes compared to control subjects. In addition, the relative activity between arginase and NOS was quantified by determination of the end-product of each enzyme as a ratio of each other, i.e the ornithine/citrulline-ratio. Subjects with diabetes had a significantly higher ornithine/citrulline-ratio, which indicates that these subjects have a higher arginase activity in relation to NOS activity compared to the control subjects. Experimental models support our finding. Arginase inhibition was evaluated in an infarct model where animals were either given saline or nor-NOHA. Plasma samples showed a clear increase in citrulline, i.e. the end-product of NOS together with a decrease in ornithine after administration of nor-NOHA compared to animals which received saline (66). The change of the amino acid ratios, citrulline/ornithine and citrulline/L-arginine, were increased in animals with arginase blockade, suggesting a relative shift towards increased NOS activity. As we only analyzed the plasma amino acid levels at baseline and not after intervention, we do not know if the amino acid levels change after intervention in **Study II**. We show that the relative activity of arginase is increased in subjects with diabetes at baseline and that they improve their microvascular endothelial function after arginase blockade. Interestingly, there was no change in L-arginine levels between subjects with and without diabetes. It is known that arginase metabolizes L-arginine, thereby competing with NOS for the same substrate. One suggested mechanism of reduced bioavailability of NO is the lack of substrate for NOS, by which arginase may play an important role. It may therefore be unexpected that we did not observed any difference in

L-arginine levels between patients with diabetes and healthy subjects. However, the samples are drawn from plasma suggesting the circulatory levels of L-arginine is satisfactory but this might not mirror the milieu in the cells. Also, L-arginine is compartmentalized in different subcellular pools that have poor redistribution (130). Thus, L-arginine availability may be limited at the subcellular compartments of importance for NO production. No additional effect have been seen in microvascular reactivity administering both arginase inhibitor and L-arginine in subjects with hypertension, L-arginine supplementation alone showed no change in microvascular reactivity (110). Further supporting the notion that arginase is of importance for endothelial function in subjects with diabetes is the observation by our lab that subjects with diabetes and macrovascular complications have increased levels of ornithine/citrulline, ornithine/L-arginine, and proline/citrulline ratios (77, 131). These ratios inversely correlate with EDV and EIDV measured with venous occlusion plethysmography in the forearm, suggesting that arginase is involved in the regulation of vascular reactivity.
There are currently no isoform-specific arginase inhibitors available. Therefore, it is difficult to determine which of the two different isoforms of arginase is of pathophysiological importance in diabetes. Romero and coworkers investigated the role of arginase in diabetic mice in which the gene for arginase II was deleted together with one allele of the arginase I gene. Wild type diabetic mice had reduced endothelial function, increased coronary fibrosis, vascular stiffness, and ROS production together with increased arginase activity and expression of arginase I (95). Heterozygote arginase I knockout mice had lower arginase activity and arginase I expression, reduced coronary fibrosis, vascular stiffness, ROS-production, and increased endothelial function (95). The response in homozygote arginase II knockout mice was similar to that observed in wild type diabetic mice. Based on these observations it was suggested that arginase I is of major pathophysiological importance in diabetes. In contrast, a knockout model of arginase II has been described to enhance relaxation in corpora cavernosa, which is largely dependent on NO. Today there is no consensus of which isoform that is the most important for the regulation of endothelial function.

Reperfusion injury

In **Study III**, the acute role of arginase inhibition on endothelial function after 20 minutes of ischemia and 20 minutes of reperfusion was investigated using FMD. IR markedly reduced endothelial function in patients with CAD during administration of NaCl. In contrast, arginase inhibition completely prevented the IR-induced reduction of FMD. These observations are supported by the findings in experimental studies. Jung and co-workers demonstrated a reduction of infarct size by 50% after iv administration of nor-NOHA in an experimental IR rat model of myocardial IR (66, 112). In contrast, iv administration of nor-NOHA did not decrease infarct size in pigs subjected to 40 minutes of ischemia and 4 hours of reperfusion. However, local intra-coronary infusion of nor-NOHA (2 mg/min for 15 min) resulted in significantly decreased infarct size, similar to the effect observed in rats (132). In our experiment, local nor-NOHA infusion (0.1 mg/min for 20 min) improved endothelial function in CAD patients. These observations suggest that if a clinical trial was to take place, the administration of an arginase inhibition should be administered locally i.e. by intra-coronary infusion during the percutaneous coronary intervention and preferably starting before opening of the occluded vessel. The latter is based on the protocols of pre-clinical studies examining cardioprotection and our protocol in **Study III** in which administration of nor-NOHA was initiated before the onset of reperfusion.

It is of importance to evaluate interventions against IR injury in patients with co-morbidities such as diabetes (113) since the efficacy of protective regimens have been demonstrated to be affected by co-morbidities (133) and since patients with diabetes have poorer outcome after myocardial infarction (60). Surprisingly, in our observations, subjects with diabetes did not decrease in FMD in response to IR. The reason for this is unclear. However, in the diabetes group, FMD was improved to levels above baseline in response to arginase inhibition despite the period of IR. This is of great importance as it seems to be more difficult to successfully treat IR injury in animals with diabetes (134). One explanation of our findings of improved FMD after arginase inhibition, could be that the baseline endothelial function of diabetic patients is poor and that arginase inhibition improves baseline endothelial function in line with the results from **Study I**. However, in **Study I** it took 120 min before a significant difference in endothelial function could be seen which suggest that the improvement by nor-NOHA in **Study III** is of acute nature and therefore related to the IR injury rather than that the baseline endothelial function is improved. Collectively, the data suggest that arginase inhibition improve FMD after IR in patients with CAD with and without diabetes.

In the clinical setting, arginase I has been shown to be upregulated in healthy subjects after global hypoxia (129). Furthermore, the same study observed that subjects resuscitated cardiac arrest had higher plasma arginase I than healthy control subjects. This is further supported by the observations that arginase activity was significantly increased in the myocardium following IR in rats subjected to myocardial IR (69). The difference was present 20 min and 8 days after reperfusion. Furthermore, arginase inhibition decreased arginase activity in ischemic myocardium compared to vehicle, both after 2 h and 8 days of reperfusion (69). The same study also provides evidence for decreased infarct size after arginase inhibition both in the acute setting and after eight days, suggesting a long-term effect despite the acute administration. The clear beneficial effect of single administration of an arginase inhibitor in IR injury in experimental animals and in patients with CAD demonstrated in **Study III** should encourage the design and initiation of a clinical trial evaluating the beneficial effect of arginase inhibition to reduce infarct size in patients with ST-elevation myocardial infarction.

Challenges in translating therapies of cardioprotection to the clinic

A great number of cardioprotective therapies have been proven to reduce infarct size in experimental models, although the evidence showing clinical benefit in patients is scarce (135). The reason for this is unclear and several possible mechanisms might be involved. One important factor may be the confounding effect of co-morbidities. For example, the cardioprotective effect of remote ischemic conditioning is attenuated or absent in diabetes (133, 136). It is therefore of importance to investigate therapeutic strategies in patients or animal models with relevant co-morbidities. It is if interest that arginase inhibition improved endothelial function following IR in patients with CAD and diabetes in **Study III**. Another factor that may influence the therapeutic effect of protection against IR injury is other medication that the patients are given. Our observations provide evidence that arginase inhibition improves endothelial function beyond that induced by other therapies given such as ACE inhibitors and statins. The timing and route of intervention may be of importance. In our study, the arginase was given by i.a. infusion starting before the onset of reperfusion. It is possible that insufficient local concentration of the drug is achieved if given systemically.

Collectively, data obtained from the present study suggest that arginase inhibition may represent an attractive therapeutic strategy to protect against IR injury in the presence of co-morbidities and in addition to other cardiovascular medications.

Arginase inhibition, involvement of red blood cells in IR

We have observed improved endothelial function in response to the administration of arginase inhibitor after IR. In experimental studies, decreased infarct size has been reported repeatedly in animals treated with arginase inhibition compared to control animals (66, 69, 132). However, the cellular source of the targeted arginase in this setting is unclear. Hitherto, functional arginase was thought to be expressed mostly by endothelial cells, where the majority of NO production takes place. However, red blood cells (RBCs) express vast amounts of arginase I, and recent data suggest that RBCs may be of great importance in myocardial IR. Yang and coworkers demonstrated a protective role of arginase inhibition in an isolated rat heart subjected to global ischemia and reperfusion (137). Interestingly, this protection was seen only in the presence of RBCs. Furthermore, the protective effect of arginase inhibition was completely dependent on RBC eNOS. These findings suggest a new mechanism for the communication between the RBCs and endothelium/myocardium, whereby RBC arginase regulates eNOS to mediate the protective effect mediated by arginase inhibition. The functional significance of RBCs for heart function in diabetes was also recently demonstrated. RBCs from diabetic mice given to wildtype hearts resulted in reduced recovery after IR compared to RBCs from wildtype mice (138). This reduction in heart recovery in diabetic mice could be counteracted by administration of NOS or arginase inhibitors. Together these observations suggest an important role of arginase in RBCs for the action of arginase inhibition in cardioprotection.

The effect of arginase inhibition on endothelial function in patients with FH

LDL is crucial for the development of atherosclerosis and has been linked to up-regulation of arginase. It is therefore of great interest to evaluate endothelial function and the role of arginase inhibition in patients with high levels of LDL. Hence, we included patients with FH to evaluate the effect of local arginase inhibition on endothelial function *in vivo*.

Patients with FH did not have reduced EDV at baseline compared to healthy controls. The lipid-lowering treatment was discontinued for 4 weeks which resulted in a prominent increase in LDL levels among patients with FH from 4.3±0.9 to 7.6±1.9 mmol/l (P<0.001). Basal EDV was not impaired despite the significance increase in LDL levels in patients with FH. This is in contrast to reduced EDV described among patients with hypercholesterolemia already after 2 weeks without lipid-lowering drugs (58).

Patients with FH had a significantly greater improvement in EDV after arginase inhibition compared to control subjects, regardless if the subjects with FH received lipid-lowering drugs or not. Holowatz and co-workers evaluated cutaneous dilatation in hypercholesterolemic subjects before and after atorvastatin treatment (127). During hypercholesterolemic conditions cutaneous vasodilatation was reduced. They further showed an improvement after cutaneous administration of an arginase inhibitor by microdialysis. The same study showed

increased arginase activity in skin biopsies collected from hypercholesterolemic subjects, compared to healthy controls but also compared to skin biopsies from the same patients after 3 months of atorvastatin treatment. Collectively these data provide supporting evidence that arginase is of importance for regulation of endothelial function in hypercholesterolemic subjects. In **Study IV**, arginase inhibition improved EDV both in FH with and without lipid-lowering drugs but also among control subjects. This was a surprising finding because in **Study I** we did not observe an increase in EDV in response to arginase inhibition among healthy subjects. The interpretation of this finding is complex. On the other hand, all groups in **Study IV** improved in EDV but the improvement in the FH group in response to arginase inhibition was greater compared to healthy individuals, regardless whether they received lipid-lowering drugs or not. This suggests that arginase inhibition is of additional benefit in improving EDV compared to lipid-lowering drugs alone.

Interestingly, several studies have described a clear link between LDL, increased arginase activity and reduced NO bioavailability (89, 91, 108, 139) and have examined the mechanisms behind it. Of great interest is that arginase inhibition improves endothelial function measured *in vitro* with vessels taken from atherogenic mice but also from wildtype mice on high fat diet (91). Furthermore, long-term treatment with arginase inhibition decreased the frequency and thickness of the plaque in atherogenic mice (91). In experimental models of atherosclerosis and hypercholesterolemia, arginase II has been suggested to be a key regulator of ROS production, NO production, and endothelial function (91, 108). New post-translational mechanistic insights have been demonstrated by Ryoo et al. (89), and Pandley et al. (139), who showed that arginase II is translocated to the cytoplasm from the mitochondrion in response to oxLDL. The signaling of oxLDL is mediated by LOX-1 and ROCK activation downstream of the receptor (139). Moreover, statins have been shown to improve endothelial function, reduce arginase activity, and block LOX-1-dependent ROCK signaling (78, 139, 140). These mechanisms are of importance for the understanding of arginase activation in hypercholesterolemic subjects. Additionally, glucose and oxLDL have been shown to increase arginase activity by activating Rho (139), suggesting a common pathway for activation of arginase. The insights gained by describing the molecular processes behind up-regulation of arginase are of importance for the understanding of the pathophysiology of arginase and the development of new possible therapies modulating arginase.

Strategies to improve NO bioavailability

The beneficial effect of arginase inhibition on the vascular bed seems to be mediated by increased NO production as the improvement in endothelial function was blocked by administering a NOS-inhibitor as demonstrated in **Study I**. If arginase inhibition works through NOS and increased bioavailability of NO, can't we just administer NO to exert a similar effect? This has been investigated previously, although with negative results. Chronic treatment with organic nitrates have been show to induce nitrate tolerance and endothelial dysfunction (141). A similar finding was seen in patients with acute lung injury where the positive response to inhaled NO disappeared already after a few days of treatment (142). Two phase II trials have evaluated intra-venous and intra-coronary nitrite administration in patients with acute myocardial infarction (143, 144). None of them were able to demonstrate a reduction in infarct size. Although Jones et al. demonstrated reduced adverse events after 1 year in the nitrate group (143). There are other approaches that have been used to try to

increase NO levels. One is administration of the substrate of NOS, L-arginine. The idea that administration of L-arginine would be able to increase NO production despite the fact that the concentration in cells is 0.1-2 mM, in the context of the Km of NOS which is 3-6 μM (145), is referred to as the L-arginine paradox (146). In addition, the Km for arginase (5 mM) is lower than that of NOS. However, the maximum activity for arginase far exceeds that of NOS about 1000 fold (147). In the experimental setting L-arginine administration has shown promising results. In contrast, administration of L-arginine has not demonstrated beneficial effects in patients with myocardial infarction (148). Another clinical study showed a significant reduction in FMD in patients with leg claudication after 6 month of oral L-arginine supplementation compared to placebo (149). L-arginine supplementation increased plasma levels of L-arginine, but citrulline and NO levels were not affected. Wilson et al speculated that arginase up-regulation might be of importance in causing this lack of effect. However, the high levels of L-arginine would imply that the L-arginine was not extensively metabolized, at least not reflected in plasma samples. Of further importance is the observation that L-arginine treatment does not seem to improve endothelial function *in vitro* beyond that induced by arginase inhibition alone (98, 110). Another therapy tried in the clinic is administration of BH4, a co-factor for NOS, but this also demonstrated a limited effect in patients with CAD (150).

Why would arginase inhibition be more beneficial than the therapies improving NO bioavailability described above? Oxidative stress has been shown to reduce co-factors, induce uncoupling of NOS, and react with NO. During a state of increased inflammation, such as in diabetes or atherosclerosis, delivery of exogenous NO will probably react with ROS, rapidly forming peroxynitrite. One problem with administration of substrate and co-substrate could be that they don't reach the NOS enzyme in the endothelial cells. L-arginine for example is divided among many subcellular compartments, with poor interchangeability (130). Arginase inhibition may improve synthesis and reduce elimination of NO through shunting of L-arginine to NOS and by reducing NOS uncoupling. The shunting of L-arginine, though arginase inhibition, might normalize the production of NO. Reduced NOS uncoupling yields decreased ROS production and oxidative stress. Arginase inhibition may exert synergistic effects to preserve the bioavailability of NO.

Improved endothelial function, does it matter?

We have demonstrated improvement in endothelial function in subjects with CAD, diabetes, and FH in response to arginase inhibition. However, one question remains unanswered. Does improvement in endothelial function actually matter? Evidence suggests that endothelial dysfunction is an independent risk factor for cardiovascular disease (10, 151). Similar results are observed in the Swedish population (152). These data suggest that the study population has an increased risk for cardiovascular events. Based on these observations therefore endothelial dysfunction was used as a surrogate clinical endpoint. However, it has not been demonstrated if improvement in endothelial function *per se* decreases cardiovascular events in any randomized controlled trials, mostly due to a of lack of agents specifically acting on endothelial function. Antihypertensive therapy (153) and statins improve endothelial function and reduced mortality from cardiovascular disease (140, 154). Collectively, much evidence suggests a connection between endothelial dysfunction and increased risk for cardiovascular disease but causality has not been evaluated in any randomized clinical trial.

Limitations

There are certain limitations associated with these studies. The size of the study groups is small, increasing the risk of type 1 and type 2 errors. However, based on pilot studies, we estimated the number of subjects needed to detect a difference in the predetermined primary endpoint, and met the estimated population size in all our studies. Furthermore, the smaller sample size makes it possible for mechanistic proof-of-concept studies. Endothelial dysfunction is a surrogate endpoint and the magnitude of difference in this endpoint is difficult to estimate. As discussed above, endothelial dysfunction is an independent predictor of the risk factors for cardiovascular events and co-varies with an improvement of these other risk factors such as LDL, suggesting it is a clinically relevant outcome to measure. In this thesis endothelial function has been evaluated in the arteries of the forearm, and it might be argued that atherosclerotic disease or plaque commonly causes symptoms in the legs, heart, and/or brain. However, studies evaluating endothelial dysfunction in both the heart and the arm have shown a clear correlation of endothelial function measured between the two vascular beds (155). Furthermore, atherosclerosis is a systemic disease, even if people rarely have flow-limiting plaque formation in the arm, the disease is nevertheless still present. A drawback is that there is no clear evidence fin support of which isoform of arginase is responsible for the improvement in endothelial function, as the substance nor-NOHA is an unselective arginase blocker and no selective arginase inhibitor is currently available. These studies are limited to local and short-term administration of the arginase inhibitor in the brachial artery whereas oral administration is needed to evaluate its efficacy as a long-term treatment. Since arginase is of great importance in the liver, there is the possibility that inhibiting liver arginase may risk inducing hyper ammonia if high doses of arginase inhibition are administered. However, the levels of arginase in the liver far exceed those found in the vasculature, hence, the small doses of arginase inhibition effecting the vasculature would probably have a minimal effect in the liver. A few studies have evaluated the safety of long-term arginase inhibition and so far no adverse events have been reported (109, 156). Although the methods employed in **Studies I-IV** are user-dependent, by using qualified, trained technicians variation could be kept to a minimum.

Future perspective

Arginase as an enzyme has been known for long but the research field of arginase as a therapeutic target is in its infancy. Much evidence suggests a central role of arginase for regulation of endothelial function, yet many questions remain unanswered. Which isoform of arginase is of greatest pathophysiologic importance? What role does arginase have in RBCs and how does it affect homeostasis? Will arginase inhibition improve clinical outcome for patients with increased cardiovascular risk? These are a few of the areas where knowledge is lacking, and in great need of further research. Of course, additional studies need to be performed to confirm the results of the work of this thesis. In addition, larger and long-term studies with clinical end-points need to be performed before we can establish the true benefit of arginase inhibitors.

In our laboratory, new studies investigating the role of arginase for endothelial function at different levels of glycemic control in patients with diabetes are currently ongoing. In addition, the role of arginase in RBCs and the effect on endothelial function is being investigated. Furthermore, arginase has been suggested as playing a role in endothelial dysfunction in obese subjects (157), which is of great interest as there is an epidemic of obesity in today's society (158).

Arginase inhibitors are starting to be developed for clinical use. For example, in the field of cancer research arginase inhibition has been suggested to decrease tumors and is currently under clinical investigation. One study is recruiting subjects with advanced cancer diagnosis to investigate the safety and tolerability of oral administration of the arginase inhibitor CB-1158 (clinicaltrials.gov, accessed; 2017-03-13). This is of great interest as dose and toxicity are pressing concerns for all newly developed drugs. However, the dose yielding effect on tumors might be greatly different from the dose to be used in treatment of cardiovascular disease. Several companies have variants of arginase inhibitors but few have the focus to develop them with the indication of cardiovascular disease. Communications between the academia and industry have resulted in possible projects which might speed up the development of arginase inhibitors for cardiovascular disease.

Diseases caused by atherosclerosis are still the most frequent cause of death and disability worldwide. As endothelial dysfunction is a key mediator of atherosclerosis, perhaps the onset of clinical manifestation may be postponed or even hindered by improving impaired endothelial function. As patients with diabetes have reduced endothelial function and do not show significant reduction of cardiovascular mortality from classical glucose-lowering drugs, new therapies are greatly needed for this patient group, arginase inhibition could be one potential treatment. This is supported by the data presented in this thesis as arginase inhibition improves endothelial function in three different vascular beds in subjects with diabetes (Figure 17). Furthermore, if we could translate the result of cardioprotection, observed in experimental studies, into the clinical setting, then patients with myocardial infarction might have a better outcome.

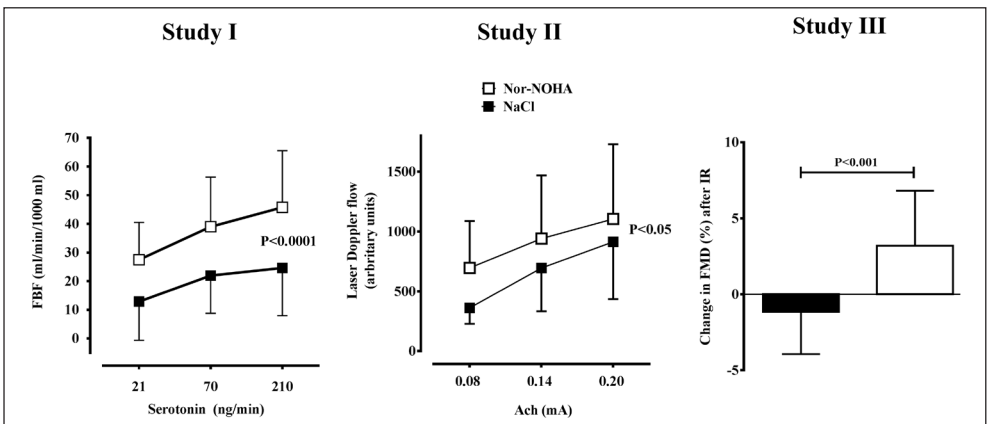

Figure 17. Evaluation of endothelial function in subjects with diabetes in three different vascular beds. In **Study I**, endothelial-dependent vasodilatation (EDV) was measured in resistance vessels with venous occlusion plethysmography before and after 120 minutes administration of nor-NOHA, N^{ω}-hydroxy-nor-L-arginine (nor-NOHA). Nor-NOHA improved in subjects with CAD+diabetes (n=16). In **Study II**, microvascular endothelial function was measured with laser Doppler flowmetry before and after 120 minutes administration of nor-NOHA. Microvascular endothelial function was improved in subjects with diabetes and microangiopathy following administration of nor-NOHA (n=12). In **Study III**, flow-mediated vasodilatation (FMD) was used to evaluate conduit artery endothelial function before and after a period of 20 minutes ischemia and 20 minutes reperfusion. Nor-NOHA improved FMD compared to administration of NaCl in patients with CAD and diabetes (n=12). Collectively, administration of nor-NOHA improved endothelial function regardless of type of vascular bed. Data are presented as mean ± SD. Ach, acetylcholine; FBF, forearm blood flow; FMD, flow-mediated vasodilatation; IR, ischemia-reperfusion; mA, milliampere.

CONCLUSIONS

I. Arginase inhibition improves basal endothelial function in patients with CAD without and with type 2 diabetes through a NOS-dependent mechanism. The positive effect of arginase inhibition was especially pronounced among patients with type 2 diabetes.

II. Arginase inhibition improves microvascular endothelial function in patients with type 2 diabetes and microvascular dysfunction.

III. Arginase inhibition improves IR-mediated endothelial dysfunction in patients with CAD.

IV. Arginase inhibition improves endothelial function in patients with familial hypercholesterolemia, irrespective of cholesterol levels.

Collectively, these results demonstrate the importance of arginase for endothelial function in patients with CAD, type 2 diabetes, and hypercholesterolemia. Arginase is thus a promising therapeutic target in the future treatment of endothelial dysfunction in patients with CAD, type 2 diabetes, or hypercholesterolemia.

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